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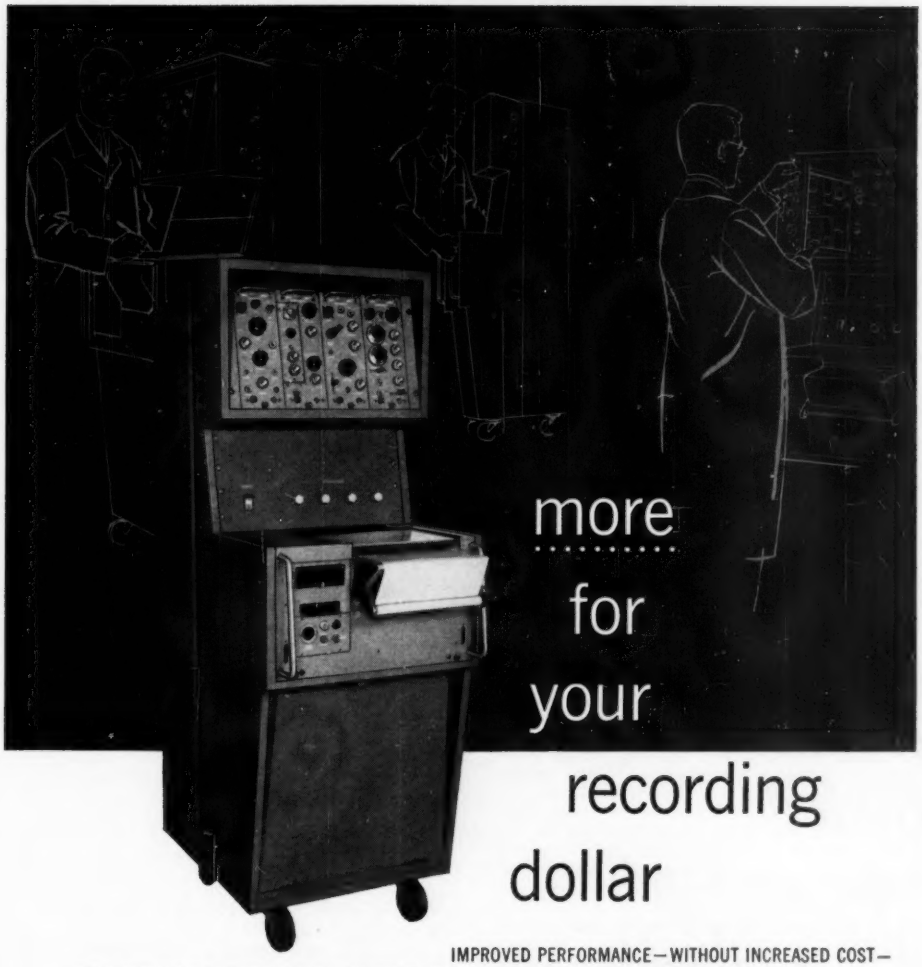

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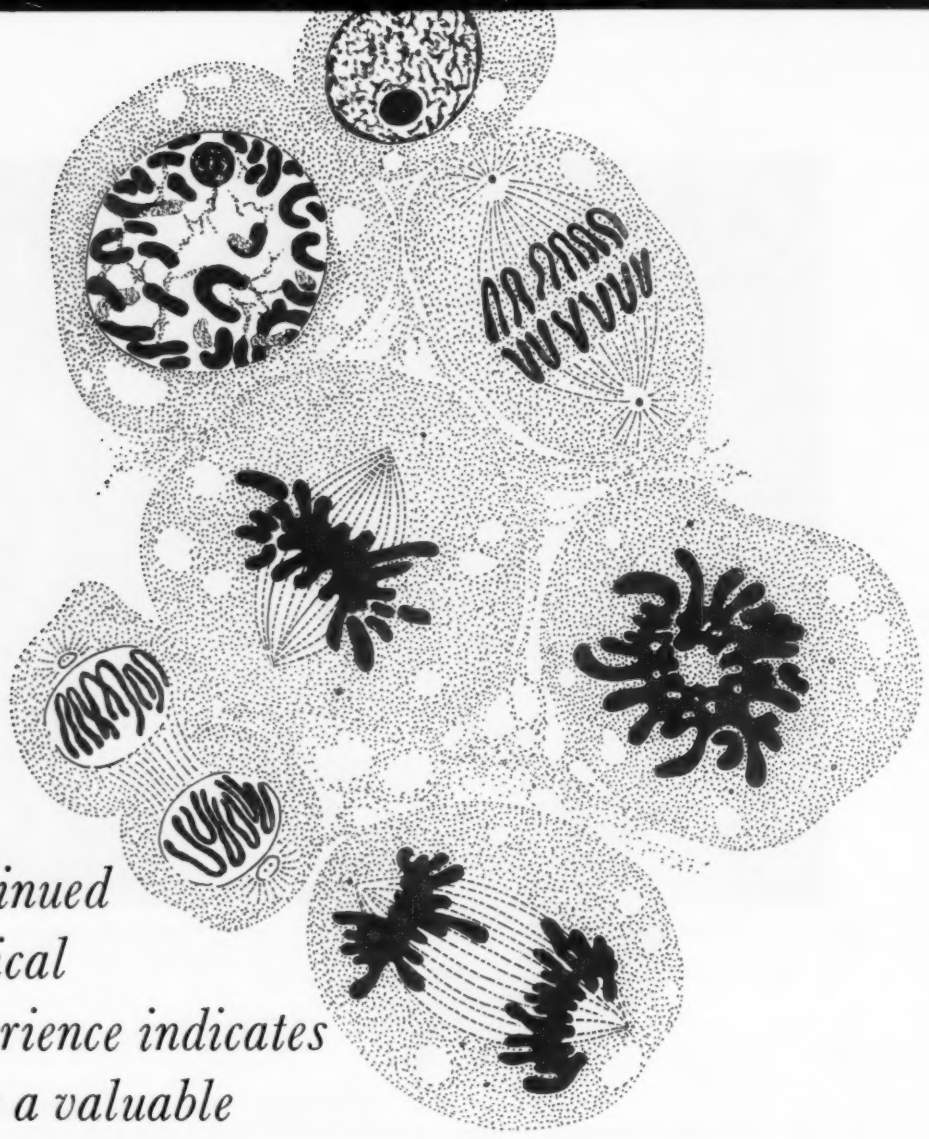

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*Papac, R.; Petrakis, N. L.; Amini, F., and Wood, D. A.: J.A.M.A. *172*:1387-1391 (March 26) 1960.

DOSAGE: *For neoplasms relatively susceptible to Cytoxan* — Patients with lymphomas and other neoplasms believed to be relatively susceptible to Cytoxan therapy are given an initial dose of 2-3 mg./Kg./day intravenously. White blood counts and platelet determinations should be made daily or twice weekly and the dosage adjusted accordingly. Intravenous infusions should be continued for at least 6 days unless otherwise indicated. A leukopenia of between 1500 and 5000 cells per cu. mm. (or lower) may be expected between the tenth and fourteenth day. In the presence of a leukopenia of less than 2000/cu. mm. Cytoxan should be discontinued until the white cell count returns to 2000 to 5000 (usually within a week). Dosage is subsequently adjusted as indicated by the patient's objective response and the leukocyte count. If the patient is subjectively improved, if the size of the tumor has decreased, or if the white cells are satisfactorily maintained between 2000 and 5000/cu. mm. oral dosage may be instituted equivalent to intravenous dosage.

Thrombocytopenia is rarely observed on this regimen. If platelet counts of less than 100,000/cu. mm. are observed, the patient should be watched carefully. If platelets continue to decrease, Cytoxan should be discontinued.

The patient who has had previous treatment with alkylating agents, or x-ray, or is debilitated may be more susceptible to bone marrow depression, and initial Cytoxan doses should be more conservative than the above. Such patients should have more frequent hematologic evaluation. Good medical practice demands access to a reliable hematologic laboratory when using Cytoxan.

For neoplasms relatively resistant to Cytoxan — Patients with carcinomas and other malignant neoplasms believed to be less susceptible to Cytoxan therapy are given a dose of 4 to 8 mg./Kg./day intravenously. Unless there are indications to the contrary, this dose is continued for 6 days, then stopped. Leukopenia usually ensues on the tenth to fourteenth day after the first dose of Cytoxan. Thrombocyte reduction is not common, and platelets may actually increase. The leukocyte count promptly returns toward normal levels in most cases, and as it begins to increase, sufficient Cytoxan is administered to maintain it near 2000 to 5000/cu. mm. This may be accomplished by two intravenous injections weekly, or by oral administration, or by a combination of both routes. An oral dosage of 50 to 200 mg. daily or an intravenous injection of 5 mg./Kg. twice weekly will usually suffice.

The platelet and leukocyte counts should be followed carefully, and the prior treatment history of patients carefully evaluated as delineated above.

Leukopenia as a guide to adequacy of dosage — The best objective measure for dosage seems to be the number of circulating white blood cells. This is used as an index of the activity of the hematopoietic system, especially the bone marrow. The mechanism by which Cytoxan causes a reduction in the level of white blood cells is not known, but cessation of dosage results in an increase in the level, indicating that the hematopoietic system had not been permanently affected. When large doses (8 mg./Kg./day for 6 days) are given initially, the white cell count falls rapidly. Following the cessation of the 6-day course, the white cells may continue to decline for as long as 8 days and then increase. The reduction of the white cell count during Cytoxan therapy and its subsequent increase when therapy is discontinued can be repeated in the same patient. Maximal reduction in leukocyte count indicates the maximal permissible Cytoxan level for therapeutic effect. Leukopenic patients must be watched carefully for evidence of infection.

Total white blood cell and thrombocyte counts should be obtained 2 or more times weekly in order to evaluate therapy and to adjust dosage.

SIDE EFFECTS: Although Cytoxan is related to nitrogen mustard, it has no vesicant effect on tissue. It does not traumatize the vein when injected intravenously, nor does it cause any localized tissue reaction following extravasation. It may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally or directly into the tumor, when indicated. It is apparently active by each of these routes.

Nausea and vomiting are common and depend on dose and on individual susceptibility. However, many investigators accept the nausea and vomiting in favor of maintaining maximal therapy. The vomiting can be controlled with antiemetic agents.

Alopecia is a frequent side reaction to Cytoxan therapy. It has been observed in 28% of the patients studied in this country. The incidence is greater with larger doses. The loss of hair may first be noted about the 21st day of therapy and may proceed to alopecia totalis. This effect is reversed following discontinuance of Cytoxan; during reduced maintenance therapy, hair may reappear. It is essential to advise the patient in advance concerning this effect of the drug.

Dizziness of short duration and of minor degree has occasionally been reported.

Leukopenia is an expected effect and can be used as a guide to therapy. Thrombocytopenia may occur, especially after large doses. The leukocyte or platelet counts of an occasional patient may fall precipitously after even small doses of Cytoxan, as with all alkylating agents. The drug should be discontinued in such patients and reinstituted later at lower dosage after satisfactory hematologic recovery has occurred. Prior treatment with x-ray or with other chemotherapeutic agents frequently causes an earlier or exaggerated leukopenia or thrombocytopenia after Cytoxan medication. Only rarely has there been a report of erythrocyte or hemoglobin reduction.

ADMINISTRATION: Add 5 cc. sterile water (Water for Injection, U.S.P.) to 100 mg. of Cytoxan in the sterile vial (add 10 cc to 200 mg. vial). Shake, allow to stand until clear, remove with sterile syringe and needle and inject.

The freshly prepared solution of Cytoxan may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally, or directly into the tumor. The solution should be administered promptly after being made but is satisfactory for use for three hours after preparation.

If the patient is receiving a parenteral infusion, the Cytoxan solution may be injected into the rubber tubing if the solution is glucose or saline.

No thrombosis or thrombophlebitis has been reported from injections of Cytoxan. Extravasation of the drug into the subcutaneous tissues does not result in local reactions.

PRECAUTIONS: Cytoxan should not be given to any person with a severe leukopenia, thrombocytopenia, or bone marrow infiltrated with malignant cells. It may be given with suitable precautions to patients who have had recent x-ray treatment, recent treatment with a cytotoxic agent, a surgical procedure within 2-3 weeks, or debilitated patients.

AVAILABILITY: Cytoxan is available as follows:

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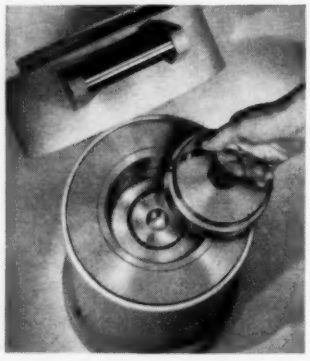

2.

1. Counterbalanced lid raises easily. Small central plug in lid can be removed if test tubes longer than 150 mm are used. Plug can be left in place and lid lowered on tubes up to 150 mm long. Splash guard protects crystal and shielding from radioactive spills.

2. Top view. Lead ring is removed to expose top of crystal. Bottles up to 5½" in diameter can be positioned directly on top of the crystal (see figure 3).

3.

4.

3. Bottle positioned in shield. Large plug in lid has been removed so that cover can be closed around bottle.

4. Lead ring surrounding the crystal can also be removed so that re-entrant type containers can be placed around the crystal.

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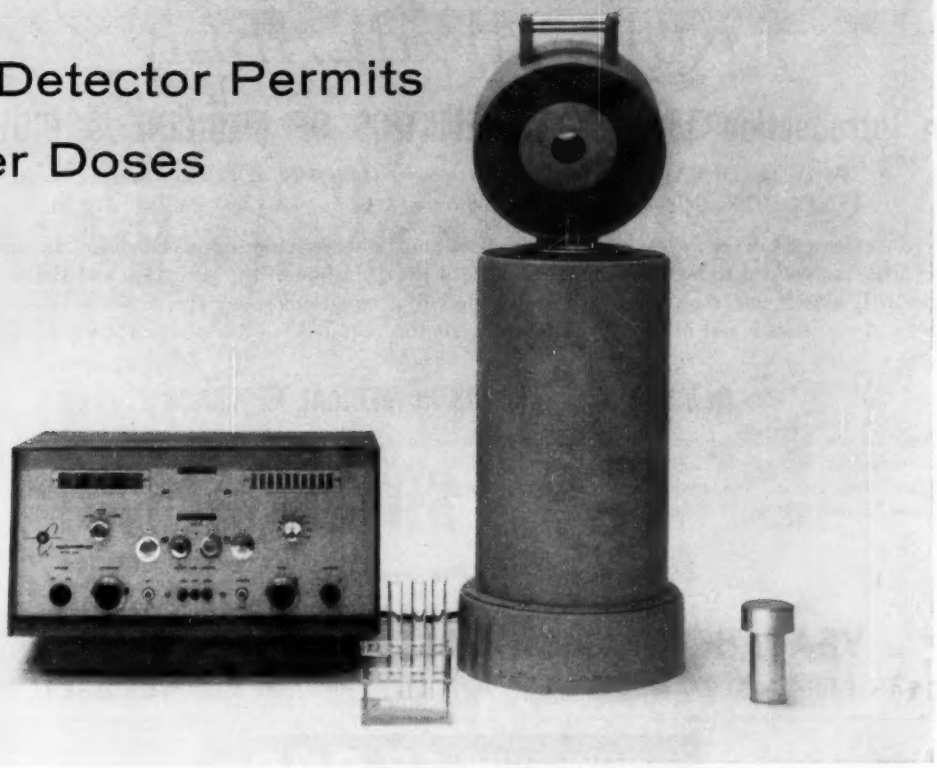

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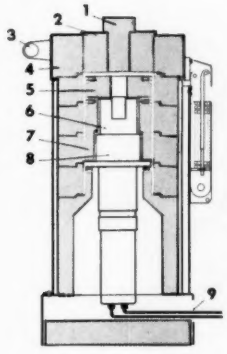

Cross-section of DS-303 Well Detector

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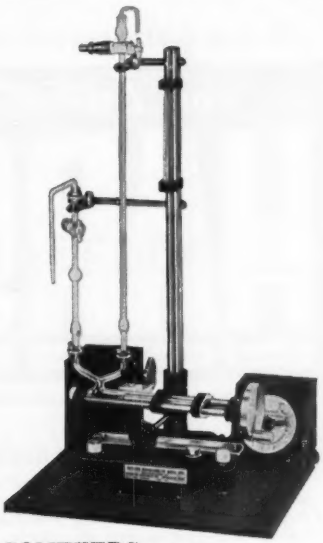

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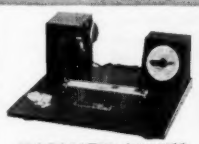

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In vitro studies show that STAPHCILLIN is a bactericidal penicillin with activity against staphylococci resistant to penicillin G. Strains of staphylococci so far tested have been sensitive to STAPHCILLIN *in vitro* at concentrations of 1-6 mcg. per ml. These levels are readily attained in the blood and tissues by administration of STAPHCILLIN at the recommended dosage. This unique attribute is probably due to the fact that STAPHCILLIN is stable in the presence of staphylococcal penicillinase. STAPHCILLIN also resists degradation by *B. cereus* penicillinase. The antimicrobial spectrum of STAPHCILLIN with regard to other microorganisms is qualitatively similar to that of penicillin G; but considerably higher concentrations of STAPHCILLIN are required for bactericidal activity than is the case with penicillin G.

STAPHCILLIN is rapidly absorbed after intramuscular injection. Peak blood levels (6-10 mcg./ml. on the average after a 1.0 Gm. dose) are attained within 1 hour; and then progressively decline to less than 1 mcg. over a 4 to 6 hour period. It is poorly absorbed from the gastrointestinal tract. STAPHCILLIN is rapidly excreted by the kidney.

As shown by animal studies, STAPHCILLIN is readily distributed in body tissues after intramuscular injection. Of the tissues studied, highest concentrations are reached in the kidney, liver, heart and lung in that order; the spleen and muscles show lower concentrations of the antibiotic. STAPHCILLIN diffuses into human pleural and prostatic fluids, but its diffusion into the spinal fluid has not yet been completely studied. However, one patient with meningitis showed a significant concentration in his spinal fluid while on STAPHCILLIN therapy.

Toxicity studies with STAPHCILLIN and penicillin G in animals show that they have approximately the same low order of toxicity.

Certain staphylococci can be made resistant to STAPHCILLIN in the laboratory, but this resistance is not related to their penicillinase production. During the clinical trials, no STAPHCILLIN-resistant strains of staphylococci were observed or developed; the possibility of the emergence of such strains in the clinical setting awaits further observation.

PRECAUTIONS

During the clinical trials, several mild skin reactions, e.g., itching, papular eruption and erythema were observed both during and after discontinuance of STAPHCILLIN therapy. Patients with histories of hay fever, asthma, urticaria and previous sensitivity to penicillin are more likely to react adversely to the penicillins. It is important that the possibility of penicillin anaphylaxis be kept in mind. Epinephrine and the usual adjuvants (antihistamines, corticosteroids) should be available for emergency treatment. Because of the resistance of STAPHCILLIN to destruction by penicillinase, parenteral *B. cereus* penicillinase may not be effective for the treatment of allergic reactions. Information with regard to cross-allergenicity between penicillin G, penicillin V, phenethicillin (Syncillin) and STAPHCILLIN is not available at present. If superinfection due to Gram-negative organisms or fungi occurs during STAPHCILLIN therapy, appropriate measures should be taken.

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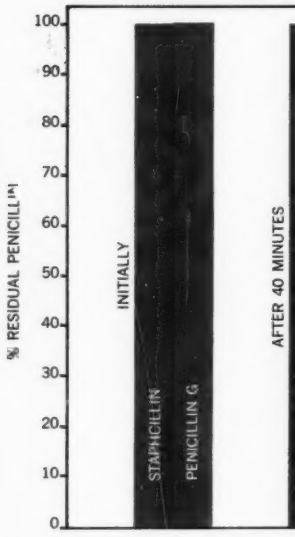

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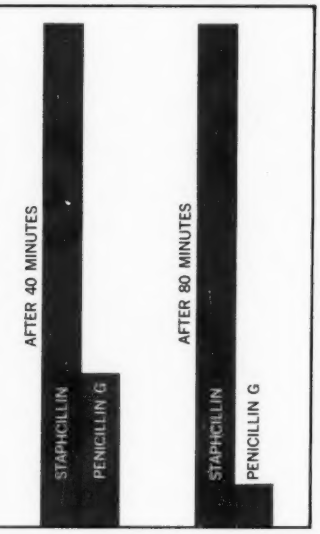

In the presence of staphylococcal penicillinase, STAPHCILLIN remained active and retained its antibacterial action. By contrast, penicillin G was rapidly destroyed in the same period of time. *(After Gourevitch et al., to be published)*

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sodium dimethoxyphenyl penicillin
FOR INJECTION

al infections to respond to penicillin therapy is attributed to zyme, penicillinase, produced by the invading staphylococcus.

e because it retains its antibacterial activity despite the pres- illinase.

ss of STAPHCILLIN has been confirmed by dramatic results in due to "resistant" staphylococci, many of which were serious

ignificant systemic toxicity. It is well tolerated locally, and jection site is comparable to that following the injection of *cases, typical penicillin reactions may be experienced.*

TION SERVICE – The attached Official Package Circular provides com- ations, dosage, and precautions for the use of STAPHCILLIN. If you desire ing clinical experiences with STAPHCILLIN, the Medical Department of ervice. You may direct your inquiries via collect telephone call to New York, Medical Department, Bristol Laboratories, 630 Fifth Ave., N. Y. 20, N. Y.

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November, 1960

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(sodium dimethoxyphenyl penicillin)
For Injection

DESCRIPTION

STAPHCILLIN is a unique new synthetic parenteral penicillin produced by Bristol Laboratories for the specific treatment of staphylococcal infections due to resistant organisms. Its uniqueness resides in its property of resisting inactivation by staphylococcal penicillinase. It is active against strains of staphylococci which are resistant to other penicillins.

Each dry filled vial contains: 1 Gm. STAPHCILLIN (sodium dimethoxyphenyl penicillin), equivalent to 900 mg. dimethoxyphenyl penicillin activity.

INDICATIONS

STAPHCILLIN is recommended as specific therapy only in infections due to strains of staphylococci resistant to other penicillins, e.g.:

Skin and soft tissue infections: cellulitis, wound infections, carbuncles, pyoderma, furunculosis, lymphangitis and lymphadenitis.

Respiratory infections: staphylococcal lobar or bronchopneumonia, and lung abscesses combined with indicated surgical treatment.

Other infections: staphylococcal septicemia, bacteremia, acute or subacute endocarditis, acute osteomyelitis and enterocolitis.

Infections due to penicillin-sensitive staphylococci, streptococci, pneumococci and gonococci should be treated with Syncillin® or parenteral penicillin G rather than STAPHCILLIN. Treponemal infections should be treated with parenteral penicillin G.

DOSAGE AND ADMINISTRATION

STAPHCILLIN is well tolerated when given by deep intragluteal or intravenous injection.

As is the case with other antibiotics, the duration of therapy should be determined by the clinical and bacteriological response of the patiert. Therapy should be continued for at least 48 hours after the patient has become afebrile, asymptomatic and cultures are negative. The usual duration has been 5-7 days.

Intramuscular route: The usual adult dose is 1 Gm. every 4 or 6 hours. Infants' and children's dosage is 25 mg. per Kg. (approximately 12 mg. per pound) every 6 hours.

Intravenous route: 1 Gm. every 6 hours using 50 ml. of sterile saline solution at the rate of 10 ml. per minute.

**Warning:* Solutions of STAPHCILLIN and kanamycin should not be mixed, as they rapidly inactivate each other. Data on the results of mixing STAPHCILLIN with other antibiotics are being accumulated.

DIRECTIONS FOR RECONSTITUTION

Add 1.5 ml. sterile distilled water or normal saline to a 1 Gm. vial and shake vigorously. Withdraw the clear, reconstituted solution (2.0 ml.) into a syringe and inject. The reconstituted solution contains 500 mg. of STAPHCILLIN per ml. Reconstituted solutions are stable for 24 hours under refrigeration.

For intravenous use, dilute the reconstituted dose in 50 ml. of sterile saline and inject at the rate of 10 ml. per minute.

*This statement supersedes that in the Official Package Circulars dated September and/or October, 1960.

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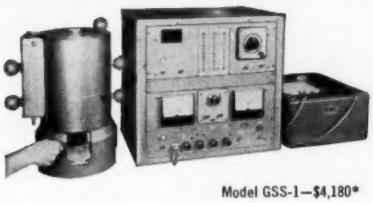

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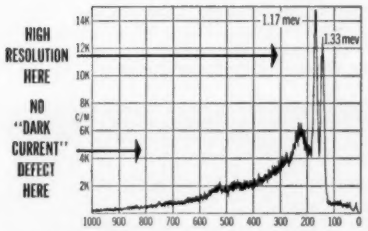
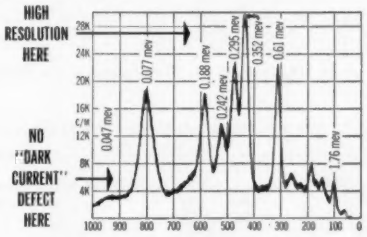


Actual spectrogram of Cobalt-60 made with a GSS-1 gamma spectrometer system. Window No. 3. Time constant: 2 sec.



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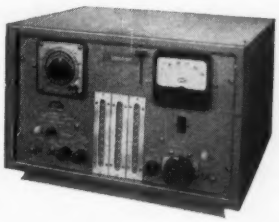

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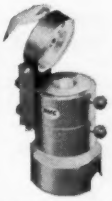

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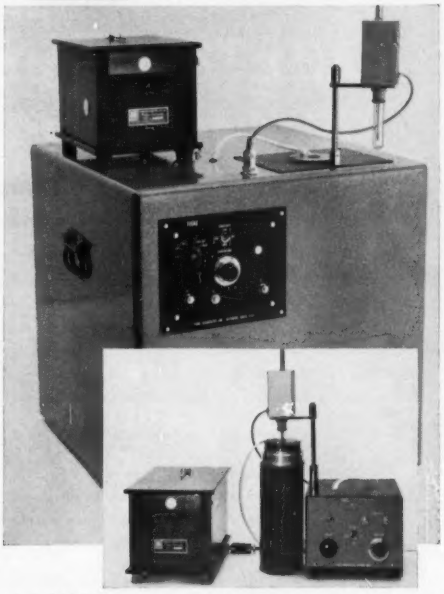

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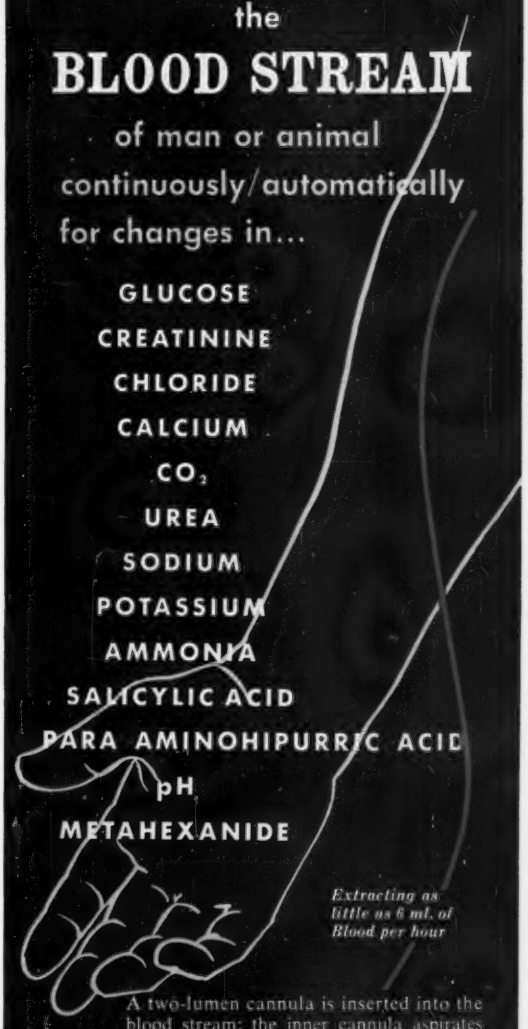

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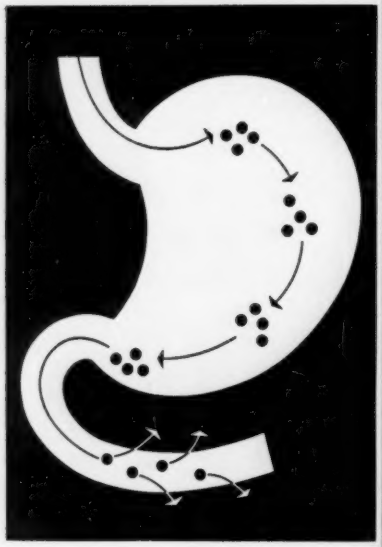

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(From The Rockefeller Institute, New York, N. Y.)

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Wilson's disease, a rare heritable disorder of copper metabolism, has been the subject of considerable investigation in recent years, and a number of interesting clinical and biochemical facts (1–4) have become known. However, the exact pathogenesis and perhaps even the fundamental metabolic defect involved remain to be elucidated. The inheritance pattern is that of a rare recessive autosomal gene (5, 6) with only the homozygous individual manifesting symptoms. It is clear that there exists a state of positive copper balance, since increased quantities of copper have accumulated in various tissues of the body by the time symptoms appear (7–10). Decreased fecal excretion of orally or intravenously administered copper (11), increased urinary excretion of copper (12), and diminished total serum copper (5, 13) characteristically are detectable. Special attention has been directed toward ceruloplasmin, a blue copper-containing serum protein with oxidase activity which migrates electrophoretically as an α_2-globulin (13, 14). The quantity of this protein is greatly decreased in the serum of almost every patient with Wilson's disease. Several investigators consider the deficiency of this protein to be the direct result of an abnormal gene as well as the basic defect of the disease (15). A decreased incorporation of radioactive copper into ceruloplasmin has been noted in patients with this disease (11, 16, 17) as well as in some heterozygotes (18); some of the latter have a decreased serum ceruloplasmin concentration (1, 4). The biological role of this protein has not yet been clarified.

In the normal individual both total serum copper and ceruloplasmin levels increase during pregnancy (19–23) as well as during estrogen administration (24, 25). A similar increase has been shown to occur in Wilson's disease (1, 4, 26, 27) following administration of estrogens. The present study, in which 11 patients were given estrogen, had a dual objective: *1*) to determine whether therapeutic benefit follows the elevation of ceruloplasmin; and *2*) by altering in a normal direction certain parameters characteristic of the genetically abnormal state, to obtain further information concerning the disturbance of copper metabolism in Wilson's disease. During the prolonged period of the study, serum copper and ceruloplasmin levels, urinary copper excretion, and clinical status of the patients were observed.

METHODS

Twenty-four-hour urine specimens were collected in acid-cleaned vessels and were stored at 4° C using thymol crystals as preservative. Urine and serum copper determinations were carried out in duplicate by methods previously described (28, 29). Serum ceruloplasmin was estimated either by the method of Bearn and Kunkel (29) and Holmberg and Laurell (30) or by a modification of Broman's method (31), all of which employ paraphenylenediamine as substrate. The serum copper calculated to be incorporated in ceruloplasmin was based on a copper content of this protein of 0.34 per cent (32); the copper present in the serum in excess of this was considered to be loosely bound to albumin (11) and is referred to as non-ceruloplasmin copper. Ethinyl estradiol (Estinyl, Schering Corp.), 5.0 mg daily, was administered during 16 courses to 11 patients (Table I).

Clinical material. The 11 patients with Wilson's disease in this study exhibited varying degrees of neurological and hepatic disorder; Table I (fourth column) indicates clinical severity ranging from 0 (no disease detectable) or 1 (minimally affected) to 4 (severely affected). Four individuals were ambulatory and in a stable phase of the disease (AC, FL, CP, LA); the remainder were severely disabled. Two cases had severe liver disease in addition to serious neurological manifestations; these two patients exhibited the highest pretreatment levels of ceruloplasmin (RE, VC). The symptoms in the remaining 9 cases were predominantly neurological.

Prior to the administration of estrogens, clinical and biochemical evaluation of each patient was made. Other forms of therapy [1] which might interfere with the study

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[1] Most of the patients have also received dimercaprol (BAL) (minimum dosage 2.5 mg per kg per day) or peni-

TABLE I

Clinical and biochemical response to estrogen administration of 11 patients with Wilson's disease

Case and Course #	Sex and Age	Age at Onset	Clinical Type*	Mean pre-estrogen levels: Serum Copper µg%	Mean pre-estrogen levels: Ceruloplasmin mg%	Mean pre-estrogen levels: Urine Copper mg/24hrs	Days of Estrogen	Peak levels with estrogen: Serum Copper µg%	Peak levels with estrogen: Ceruloplasmin mg%	Peak levels with estrogen: Urine Copper mg/24hrs	Urine Copper mg/24hrs: Penicillamine	Urine Copper mg/24hrs: BAL	Clinical changes during estrogen treatment
AC	F 28	12	N-2 H-1	73	8.0	0.30	22	90	8.9	0.71	4.42		Unchanged
FL	M 39	22	N-1 H-0	50	7.5	0.58	19	80	6.4	0.73	2.67	1.24	Dysarthria slightly worse
BR#1‡	F 40	13	N-4 H-1	60	6.7		14	119	10.2				Slight restlessness
BR#2	F 40	13	N-4 H-1	72	8.0		36	71	10.2				Unchanged
CP	M 30	20	N-1 H-0	41	5.2	0.11	14	50	6.0	0.37	2.28	1.28	Slight subjective weakness
LA	M 56	31	N-2 H-1	40	7.3	0.73	28	80	10.7	1.07	2.06	1.77	Increase of tremor and incoordination
SC	M 19	18	N-3 H-0	51	2.8	0.47	14	69	2.0	1.88	4.81	1.18	Slight increase in tremor and drooling
YR‡	F 41	13	N-3 H-1	52	4.8	0.83	21	87	8.4	1.50	2.00	0.90	Initial improvement. Sudden coma and hemiparesis day 22.
AD	M 32	27	N-3 H-1	99	10.1	0.44	28	154	24.0	5.26			Initial improvement in coordination. Then marked increase in belligerence, disorientation, dysphagia, incontinence.
RE	M 18	15	N-2 H-4	81	16.4	0.58	30	188	33.0	3.78	7.16		Tremor disappeared. Speech and disorientation worse.
MP#1	F 25	17	N-4 H-1	68	7.3	0.28	46	123	21.0	1.89	3.71	0.61	Slow, mild improvement of rigidity, flapping and speech
MP#2†	F 25	17	N-4 H-1	82	6.4	2.74†	30	70	11.5	3.00			Unchanged
VC#1	F 27	20	N-3 H-2	108	20.8	0.26	16	235	78.8	1.45			Improvement
VC#2	F 27	20	N-2 H-2	106	26.4	0.22	46	188	48.3	2.10			Striking improvement
VC#4	F 28	20	N-4 H-2	79	14.4		87	140	30.2	0.65	2.41	0.48	Mild improvement
Normal (mean)				126	38.5	0.05							

* Neurological or hepatic disorder prior to estrogen administration. Severity graded 0–4.
† Estrogen and penicillamine administered simultaneously
‡ Identical twins (50)

TABLE II

The effect of estrogens in 11 patients with Wilson's disease (mean levels)

	Total serum copper	Non-ceruloplasmin serum copper	Ceruloplasmin	Urinary copper
	μg%	μg%	mg%	mg/24 hrs
Pre-estrogen level	71	36	10.1	0.44
Estrogen response (peak)	116	46	20.6	1.84
Normal	100–130	0–10	38.5	<0.10

were discontinued for several weeks prior to institution of the hormone, except in the case of two patients (MP2, VC4) in whom the combined effect of estrogen and penicillamine was determined. Foods high in copper content were restricted.

RESULTS

Tables I and II summarize the biochemical status before administration of estrogen and indicate the peak biochemical response to such treatment. The various types of biochemical and clinical response observed in several of the patients are illustrated in Figures 1–4.

Total serum copper. In all but three instances (BR2, CP, MP2) the serum copper concentration rose during or immediately following estrogen. During each of six courses of estrogen there was a mild to moderate increase to levels of 69 to 119 μg per 100 ml. During seven courses the level rose to 123 to 235 μg per 100 ml (AD, RE, MP1, VC1–4); in three of these patients the level prior to treatment was in the higher range exhibited by some individuals with this disorder (81 to 108 μg per 100 ml). There was often a lag of several days following discontinuation of treatment before the serum copper concentration began to decrease.

Ceruloplasmin. Seven patients showed no significant increase in serum ceruloplasmin; all these had very low levels prior to treatment. Six of the seven, however, showed a mild to moderate rise in total serum copper. Of the four cases (AD, RE, MP, VC) who showed increase in ceruloplasmin, two had low concentrations prior to treatment; one (VC) had a fairly high level of ceruloplasmin (20.8 mg per 100 ml), and her response to estrogen was the most striking of the cases studied, rising to 78.8 mg per 100 ml by Day 13 after treatment was instituted (Figure 4).

Urinary copper excretion. Prior to treatment all ten patients (BR not tested because of incontinence) exhibited elevated cupruria, ranging from 0.11 to 0.73 mg per day. During estrogen administration three cases [FL, CP (Figure 1), LA] showed no significant change in urinary copper excretion while on estrogens.[2] Two patients approximately doubled their output [AC, YR (Figure 2)]. The remaining five patients showed marked increases in average daily urinary copper excretion in response to estrogen: that of SC increased from 0.45 to 1.71 mg; of MP from 0.36 to 1.68 mg; of VC from 0.32 to 1.85 mg (Figure 4); of RE from 0.58 to 1.9 mg; and of AD from 0.48 to 4.29 mg per day (Figure 3).

Interrelationships of serum copper, ceruloplasmin, and urinary copper. When given oral ethinyl estradiol some patients with Wilson's disease exhibited little or no change in the concentrations of serum copper and ceruloplasmin; such patients showed minimal or absent increase in urinary copper excretion and minimal clinical change (Figure 1). Some patients showed a rise in total serum copper without significant increase in ceruloplasmin, indicating therefore a rise in non-cerulo-

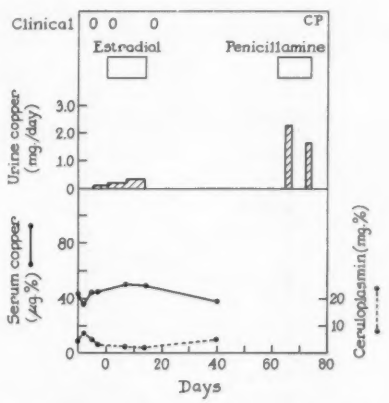

FIG. 1. RESPONSE OF PATIENT CP TO ESTROGEN, SHOWING NO BIOCHEMICAL OR CLINICAL CHANGE.

cillamine (1.0 g per day of combined *dl*-isomers, Aldrich Chemical Co., or 0.5 g per day of *d*-isomer, Distillers Co., London) but at times other than the test period; the effects of these chelating agents on urinary copper excretion are recorded in Table I.

[2] During estrogen administration (14 days) to five heterozygotes, two possible heterozygotes, and a normal male individual, significant elevation of urinary copper excretion did not occur.

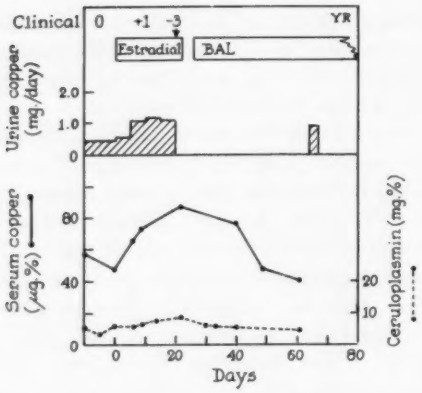

FIG. 2. RESPONSE OF PATIENT YR, SHOWING INCREASE OF TOTAL SERUM COPPER AND URINARY COPPER EXCRETION. INITIAL IMPROVEMENT FOLLOWED BY DETERIORATION.

plasmin serum copper; of this group three showed an increased cupruresis and five deteriorated neurologically (Figure 2, for example; Table III).

Four patients responded to estradiol with not only a marked increase of the serum copper to normal or supernormal levels but also an increase in ceruloplasmin content (AD, RE, MP1, VC). The ceruloplasmin levels of AD (Figure 3) and RE rose to low normal levels, but the non-ceruloplasmin copper levels of their serum rose markedly (73 and 76 μg per 100 ml). These two patients showed the greatest degree of cupruresis of all the patients as well as the most severe neurological deterioration.

MP1 initially showed a roughly parallel rise of serum copper and ceruloplasmin with moderate cupruresis and mild clinical improvement. Preceding and during a subsequent course of estrogen (MP2) penicillamine was given; the serum level of ceruloplasmin did not rise, and the serum copper level decreased to 29 μg per 100 ml in contrast to her pretreatment concentration of 68 μg per 100 ml.

VC, during her first two courses of estradiol administration (Figure 4), showed a striking rise of both total serum copper and ceruloplasmin, with moderate cupruresis. It is noteworthy, however, that during the first course the rise in total serum copper could be accounted for by the increase in the copper incorporated in serum ceruloplasmin. Thus, the amount of non-ceruloplasmin copper in the serum was relatively small. (In addition to this unique biochemical response the singular clinical response of this patient is described later.)

There was, in general, a rough correlation between the absolute height of non-ceruloplasmin

TABLE III

Biochemical and clinical alterations associated with estrogen administration in Wilson's disease *

Patients and courses	Total serum copper conc.	Ceruloplasmin conc.	Non-ceruloplasmin copper conc.	Cupruresis	Clinical signs and symptoms
AC	+	0	+	+	0
FL	+	0	+	0	+
BR#1	+	0	+		0
BR#2	0	0	0		0
CP	0	0	0	0	0
LA	+	0	+	+	+
SC	+	0	+	+	+
YR	+	0	+	+	++
AD	+	+	+	+++	++
RE	++	+	++	++	++
MP#1	+	+	+	+	0
MP#2†	−	0	0	++	0
VC#1	++	+++	−	+	
VC#2	+	++	+	+	
			0		
VC#3					
VC#4	+	+	+	+	0
			0		

* + Indicates increase; −, decrease; 0, no detected alteration.
† With penicillamine

copper and the degree of cupruresis. A parallel increase between serum copper and ceruloplasmin was frequently observed during the initial phase of the response. Subsequently, the ceruloplasmin level tended to become stable while both the total copper (and, therefore, the non-ceruloplasmin copper) and the urinary copper increased further. There was also a clinical correlation: patients whose condition deteriorated showed, in general, a high non-ceruloplasmin copper level either prior to or in response to estrogen treatment. Patients whose condition either improved or did not change showed a decrease or little change in the non-ceruloplasmin copper level.

Some patients exhibited cupruresis without a ceruloplasmin rise; however, all of those who showed cupruresis had an increase in total serum copper, which suggests that a rise in non-ceruloplasmin copper is related to an increase in urinary copper excretion.

Clinical results. Table I indicates the predominant clinical type prior to treatment and the clinical response to estrogen administration. Three cases did not change (AC, BR, CP). Three cases deteriorated slightly but returned to pretreatment status within two to three weeks following treatment (FL, LA, SC). One patient (YR) improved initially but abruptly deteriorated, developing semicoma and hemiparesis, a state very similar to that of her identical twin. However, she returned to a pretreatment status about three weeks after simultaneous discontinuation of the hormone and initiation of BAL. Two patients (AD, RE)

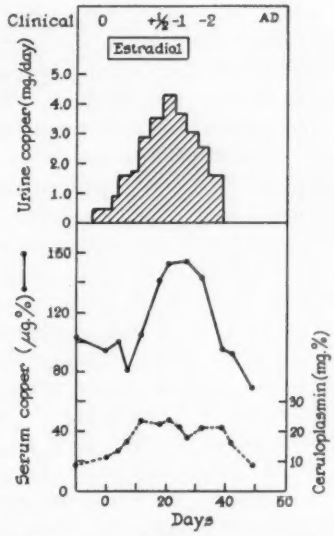

FIG. 3. RESPONSE OF PATIENT AD. INCREASE IN SERUM COPPER, MILD INCREASE IN CERULOPLASMIN, AND A STRIKING CUPRURESIS ACCOMPANIED CLINICAL DETERIORATION.

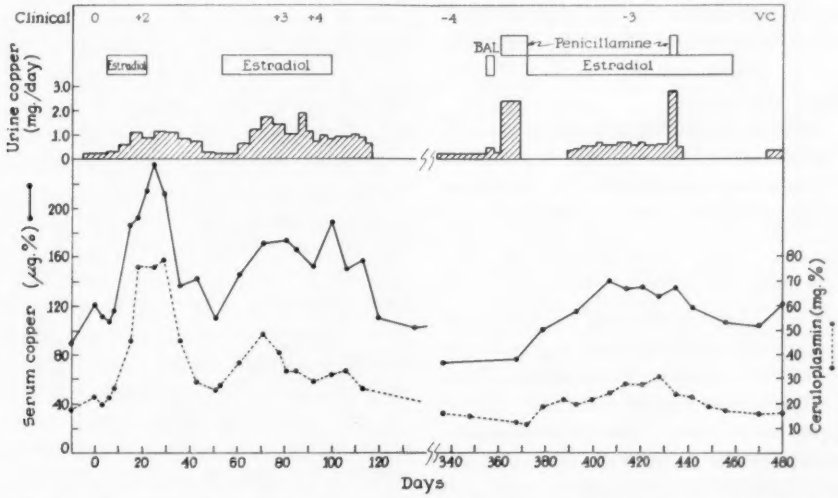

FIG. 4. RESPONSE OF PATIENT VC DURING 3 OF 4 COURSES OF ESTROGEN. During the first courses clinical improvement and only moderate cupruresis occurred in association with a great elevation of total serum copper. Much of the increase in total serum copper could be accounted for by a simultaneous increase in ceruloplasmin during course 1 and the early part of course 2.

exhibited initial neurological improvement but subsequent marked deterioration with only minimal improvement following cessation of treatment. Although both of these patients died of Wilson's disease within months following treatment, they were severely affected prior to the study.

One patient (MP) showed mild improvement during her initial course and no change during the second course which was superimposed upon penicillamine administration. VC (Figure 4) showed striking improvement when given estrogen. Prior to treatment she had become markedly debilitated, displaying severe hepatic and neurological symptoms. Prompt and extensive neurological improvement and weight gain from 35 to 47 kg occurred during the first three courses of treatment. She progressed from being bedridden to a condition in which she could be discharged from the hospital for short periods. Toward the end of each course of treatment and in the period following discontinuation of the hormone, neurological deterioration occurred. When her last course was initiated she showed severe neurological disease, and only slight improvement occurred during estrogen administration. Her biochemical response during the first courses was unique, as previously indicated. Although she is the single instance of striking improvement associated with estrogen therapy, this treatment seemed largely responsible for her survival early in the hospital course when death apparently was impending and, further, enabled her to withstand major surgery (course 3).

During estrogen administration three patients developed phlebothrombosis, in one case associated with pulmonary embolism. No consistent coagulation abnormalities could be detected.[3]

DISCUSSION

A notable lack of uniformity was evident in the response to estrogen administration of the 11 patients with Wilson's disease. It is clear (Table II) that such treatment usually leads to an alteration of copper metabolism. The nonhomogeneity in response is a further example of the clinical and biochemical variation seen in patients with this disorder (6). This, of course, does not necessarily imply genetical nonhomogeneity, but could equally represent the result of varying internal environments or compensatory physiological mechanisms, as well as modifying genes.

In many respects the patient with Wilson's disease in whom total copper and ceruloplasmin concentrations are brought toward normal by administration of estrogen is comparable with the rare patient who, at some phase of his disease, exhibits total levels in the normal range (33, 34). The reason for the increased concentration of ceruloplasmin in such unusual cases is not immediately apparent. It is noteworthy that two such patients, both males, described by Sass-Kortsak, Cherniak, Geiger and Slater (33) had severe liver disease without neurological symptoms; one showed gynecomastia and delay in maturation of male sexual characteristics. Our observations demonstrate that environmental (in this instance, endocrinal) alterations can increase the concentration of the protein, ceruloplasmin, which is characteristically present in decreased quantities in Wilson's disease.

It seems likely that some of the effects of estrogen administration to patients with Wilson's disease are the result of nonspecific mechanisms affecting protein synthesis without altering the metabolic error which is the result of the primary genetical defect. The manner in which estrogen effects anabolism in the normal individual is not clear at the present time (35–37). It is known that the concentration of a number of serum components increases during pregnancy or estrogen administration; these include fibrinogen, α_1-, α_2-, and β-globulins (38–42), ceruloplasmin (43), transcortin (44), and the thyroxine-binding α-globulin of serum (45, 46). Also, the serum copper level may rise during pregnancy (19–23, 47) or estrogen administration (24, 25, 43, 48).

When estrogen is given to patients with Wilson's disease the concentration of two distinct serum compartments may undergo alteration—*1*) non-ceruloplasmin serum copper and *2*) ceruloplasmin. Since these increases, when they occur, do not necessarily either parallel each other or occur in the same patient, the question arises of whether the hormone is influencing one metabolic mechanism only or whether the two serum components increase for unrelated reasons. The former and more attractive possibility implies that as the ceruloplasmin-synthesizing mechanism

[3] Dr. Margaret Todd of Cornell University Medical College, New York, N. Y., kindly assisted in these assays.

attempts to increase the production of this protein, simultaneous inordinate increase of the serum non-ceruloplasmin copper concentration occurs. Insufficient data exist for determining whether either of these possibilities is correct.

Since it is unknown whether alteration of the biliary or fecal excretion of copper occurs during estrogen administration, comments concerning total copper balance or actual alteration of tissue content of copper during such treatment can not be made. It is noteworthy in this respect that the normal individual remains in copper balance although he excretes virtually no copper in the urine.

Although the administration of estrogen to patients with Wilson's disease may in some instances result in increase in the serum concentration of copper and ceruloplasmin, it is not usually of therapeutic value. Although only 11 patients have been treated, and although the course of the disease is unpredictable, treatment with estrogens has been associated with increased rate of neurological decline in over half of the patients. Further, some patients in a stable phase of the disease have abruptly shown the onset of deterioration in association with administration of estrogen. Increasing neurological symptoms, increasing cupruresis, and rising non-ceruloplasmin copper concentration are all accentuations of characteristic manifestations of the preexisting disease. In the presence of this specific genetical abnormality, the efforts of the cell to increase ceruloplasmin synthesis in response to estrogen may be partially effective, as observed in 4 of 11 patients, but these efforts are usually simultaneously associated with increase in the disease processes. The lack of improvement seen in some patients whose ceruloplasmin concentrations increase is evidence that the low level of this protein per se is not critical in determining the presence or absence of the clinical and pathological findings of the disease.

Several observations made during the study thus indicate that although a trend toward normality in total serum copper and serum ceruloplasmin may be established, other events may take place simultaneously which are seriously deleterious. The increase in the non-ceruloplasmin component of serum copper (49), though associated with striking cupruresis, is also associated with increase of neurological symptoms. It is unclear whether the increase in this compartment of serum copper itself is the deleterious event or is only an associated occurrence.

SUMMARY

1. Ethinyl estradiol was administered to eleven patients with Wilson's disease. In four patients the serum copper and ceruloplasmin concentrations and the urinary copper excretion increased. In six patients there was an increase in the serum copper concentration, three of whom showed cupruresis. The levels of serum copper and ceruloplasmin and the urinary copper excretion showed no change in one patient.

2. One patient improved clinically, four were unchanged, and six deteriorated during the estrogen administration.

3. The interrelationships of total serum copper, non-ceruloplasmin serum copper, ceruloplasmin, urinary copper excretion, and clinical change were investigated. Neither ceruloplasmin elevation nor cupruresis necessarily led to improvement. Cupruresis and clinical deterioration could, in general, be correlated with an increase in the non-ceruloplasmin fraction of serum copper.

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ANNOUNCEMENT OF MEETINGS

The Fifty-Third Annual Meeting of THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION will be held in Atlantic City, N. J., on Sunday afternoon and evening, April 30, 1961, in the Chalfonte-Haddon Hall (jointly with the AFCR); and on Monday, May 1, at 9:00 a.m., at the Casino Theater on the Steel Pier.

The Eighteenth Annual Meeting of THE AMERICAN FEDERATION FOR CLINICAL RESEARCH will be held in Atlantic City, N. J., on Sunday, April 30, 1961, at 9:00 a.m., at the Casino Theater on the Steel Pier. On Sunday afternoon and evening, April 30, 1961, a joint sectional meeting with THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION will be held in rooms in the Chalfonte-Haddon Hall.

THE ASSOCIATION OF AMERICAN PHYSICIANS will hold its Seventy-Fourth Annual Meeting in Atlantic City, N. J., at the Casino Theater on the Steel Pier on Tuesday, May 2, 1961, at 9:30 a.m., and in the Carolina Room, Chalfonte-Haddon Hall, on Wednesday, May 3, 1961, at 9:30 a.m.

OXIDATIVE HEMOLYSIS AND PRECIPITATION OF HEMOGLOBIN. II. ROLE OF THIOLS IN OXIDANT DRUG ACTION *

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In a preceding report (1) it was shown that those redox compounds that cause Heinz body anemias *in vivo* produce a sequence of changes *in vitro* in solutions of crystalline hemoglobin culminating in the precipitation of hemoglobin as spherical granules. These granules appear to be identical with Heinz bodies. The sequence of changes produced in hemoglobin by these compounds includes: *1*) the formation of methemoglobin, *2*) an increase in electrophoretic and chromatographic mobility, *3*) the appearance of irreversible hemochromes such as sulfhemoglobin and *4*) frank precipitation. It was found that similar changes in hemoglobin were produced rapidly by "simple" oxidants such as ferricyanide and slowly by prolonged incubation of hemoglobin alone under oxygen. Compounds that cause Heinz body anemias apparently are converted under oxygen into redox intermediates or free radicals which catalyze the oxidative breakdown of hemoglobin, a process which proceeds spontaneously only slowly *in vitro* and presumably still more slowly during cellular aging *in vivo*.

There are many reasons to suspect the involvement of sulfhydryl (thiol) groups in the processes described above. Such groups are readily oxidized under physiologic conditions and are involved in maintaining the integrity of the red cell (2) and the physiologic activity of hemoglobin (3). The studies of Beutler and his co-workers (4, 5) have established an association between intracellular reduced glutathione (GSH) levels and the susceptibility *in vitro* and *in vivo* of red cells to injury by oxidant drugs. Based on studies of "choleglobin" formation in the presence of ascorbic acid, Mills (6) has advanced the hypothesis that GSH protects hemoglobin from oxidative breakdown by acting as a hydrogen donor in the peroxidative destruction of hydrogen peroxide. The necessity for regenerating GSH in such a mechanism would conceivably explain the hypersusceptibility to the action of oxidant compounds of red cells deficient in glucose or in glucose-6-phosphate dehydrogenase (7), since in the red cell, oxidative glycolysis via the pentose phosphate pathway (8), possibly supplemented by anaerobic glycolysis (9), provides the energy for reducing glutathione. On the other hand, there are certain differences between phenylhydrazine-like compounds and hydrogen peroxide in their action on hemoglobin (1). Phenylhydrazine and related compounds appear to destroy hemoglobin through the formation of a series of oxidant derivatives which may resemble, but are not identical with, hydrogen peroxide.

The following studies were conducted in order to explore the participation of thiol groups in the oxidative destruction of hemoglobin.

METHODS

Preparation of reagents. As described previously (1) buffered solutions of crystalline human hemoglobin were prepared by the method of Drabkin (10), and phenylhydrazine and related compounds were dissolved in isotonic (0.12 M) phosphate buffer, pH 7.4. Unless otherwise stated, glutathione,[1] an acid, was dissolved immediately before use in physiologic saline [since its reduced form is more stable at a low pH (11)] and was added slowly to buffered solutions of hemoglobin in amounts that did not affect the final pH of the solution. The sulfhydryl-binding compound, sodium *p*-chloromercuribenzoate[2] (PCMB), is a base (probably existing as sodium *p*-hydroxymercuribenzoate) that is very poorly soluble at a physiologic pH; therefore it too was dissolved in unbuffered saline as a concentrate, which was then added slowly to buffered solutions of hemoglobin.

General procedures. Methemoglobin and so-called sulfhemoglobin were measured by the Evelyn-Malloy

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[1] Schwarz Bioresearch, Inc., Mount Vernon, N. Y.

[2] Sigma Chemical Co., St. Louis, Mo.

procedure (12) as described elsewhere (1). Heinz bodies in red cells and granules of precipitated hemoglobin were stained by suspension in isotonic crystal violet. The weights of hemoglobin precipitates were determined with the aid of high speed centrifugation (1). Filter paper electrophoresis at pH 8.6 and ion-exchange resin column chromatography at pH 7.1 were carried out as reported earlier (1, 13).

Studies involving radioactive glutathione. To explore the possibility that, during the oxidation of GSH in the presence of hemoglobin, mixed disulfides may form involving GSH and the cysteinyl residues of globin, studies were made of the binding to hemoglobin of GSH labeled with either S^{35} or C^{14}.[1] Flasks containing solutions of hemoglobin were incubated at 0° or 37° C with labeled glutathione. To some of these flasks phenylhydrazine was added at the onset of the 2 hour incubation period, after which all flasks were chilled in an ice bath. As a control of the amount of labeled glutathione trapped by precipitated hemoglobin, labeled glutathione was added at the conclusion of incubation, rather than at the beginning, in some of the phenylhydrazine-containing flasks. After chilling of the specimens, quadruplicate 2 ml samples were precipitated with 6 ml of 10 per cent trichloroacetic acid (TCA) and the precipitate was washed twice with 5 per cent TCA, once with absolute ethyl alcohol, and once with ether before drying. To ascertain whether any GSH remaining with the precipitate was attached by disulfide linkage, the ability of thioglycollic acid to detach this GSH was determined. Accordingly, the dried precipitates in one pair of each quadruplicate set of tubes were dissolved in 20 per cent thioglycollic acid and those of the other pair in 40 per cent glycollic acid.[3] These solutions were allowed to stand at room temperature overnight under nitrogen, after which the protein was reprecipitated by TCA, washed, and dried as described above. The dried precipitates were weighed, dissolved in 0.1 N NaOH, and 0.1 ml samples were spread and dried on stainless steel planchets for measurement of radioactivity.

In flasks to which phenylhydrazine had been added, a portion of the contents was studied for the radioactivity of the hemoglobin precipitated by the phenylhydrazine. This precipitate was secured by cold centrifugation at 105,000 G, was dried under vacuum, dissolved with 0.1 N NaOH, and spread on planchets as described above.

Radioactivity was measured with a windowless flow counter. To obviate variations in self-absorption, all specimens, including standards, were adjusted to a uniform concentration of solids prior to sampling.

Autoradiographs of radioactive glutathione bound to hemoglobin were obtained by exposing X-ray films to filter paper electrophoretograms of the unprecipitated hemoglobin solutions dialyzed free of unbound glutathione. The electrophoretic and autoradiographic spots were quantitated with a photoelectric densitometer.

[3] This concentration of glycollic acid, used as the nonthiollic control, was selected on the basis of the relative dissociation constants of these two compounds.

Determination of glutathione. Reduced glutathione was measured by the nitroprusside method of Grunert and Phillips (14) as modified by Beutler (15). When glutathione was present in hemoglobin solutions, the hemolysis step was omitted from the procedure, the deionized water being replaced by additional 3 per cent metaphosphoric acid, saturated with sodium chloride. A standard curve was performed with each determination, using glutathione freshly dissolved in 1.5 per cent metaphosphoric acid. Such a preparation showed negligible increase in reduced glutathione after reduction at the mercury cathode (16) and gave 90 per cent of its theoretical mercury-combining groups in 0.05 M borax, 0.1 M potassium chloride, when titrated amperometrically with 1 mM mercuric chloride using the rotating platinum electrode (17).

Total glutathione was determined on a 10 ml aliquot of the metaphosphoric acid filtrate reduced over a mercury cathode in a 50 ml beaker by 40 ma of current, as described by Dohan and Woodward (16); 95 per cent reduction occurred by the end of 20 minutes, and reduction was routinely carried out 10 minutes longer.

Determination of mercury-combining sites in hemoglobin. The hemoglobin mercury-combining sites were titrated amperometrically by a modification of the method of Kolthoff, Stricks and Morren (17). A mercury-coated platinum wire electrode was used (0.5 cm long, rotated by a synchronous motor). This indicator electrode was kept -0.1 volt versus a commercial electrode (Beckman Calomel Reference Electrode, fiber type) using a manually operated circuit.

The titration of *native hemoglobin mercury-binding groups* was done in 25 ml of 0.5 M potassium chloride, 0.1 M potassium phosphate buffer, pH 7.3, at room temperature (22° to 27° C) in a 50 ml beaker. Two to 4 ml of approximately 0.1 mM hemoglobin solution, usually as its carbon monoxide derivative, was added to the buffer through which nitrogen had been bubbled. The residual current was read, then 1 mM mercuric chloride was added in 0.1 ml increments, as the solution was stirred briskly, and the diffusion current read. The whole titration required less than 5 minutes. There was no increase in galvanometer reading until the mercury-binding sites had been exceeded, allowing a sharp endpoint. It was noted that after the equivalence point was reached following additions of mercuric chloride, the diffusion current tended to fall slowly with time, probably as a result of much slower binding of mercury by other sulfhydryl groups. However, this phenomenon did not interfere with the determination of primary mercury-combining sites. The *total hemoglobin mercury-binding groups* were titrated, using denatured hemoglobin; the conditions were the same as in the titration of native hemoglobin except that the titration fluid was 8 M in urea. As a preliminary test of the electrode, prior to the addition of hemoglobin, 1 mM mercuric chloride was added to the titration fluid in 0.1 ml increments, the beaker contents stirred mechanically under nitrogen, and the galvanometer reading recorded until 0.8 ml (an excess of mercury) had been added. If the galvanometer failed to

TABLE I

Oxygen requirement for oxidation of reduced glutathione (GSH) (40 mg %) by phenylhydrazine (0.1%) and acetylphenylhydrazine (0.5%) in isotonic phosphate buffer, pH 7.4, at 37° C

Gas and additions during preincub. (3 hrs)	Gas and additions during incub. (0.5 hr)	% GSH after incub.
N_2	N_2 + GSH	100
O_2	O_2 + GSH	71
N_2 + acetylphenylhydrazine	N_2 + GSH	99
O_2 + acetylphenylhydrazine	O_2 + GSH	21
O_2 + acetylphenylhydrazine	N_2 + GSH	30
N_2	N_2 + phenylhydrazine + GSH	93
O_2	O_2 + phenylhydrazine + GSH	3
N_2	N_2 + ferricyanide + GSH	0

show a linear response to mercury, the equipment was adjusted before addition of hemoglobin. Usually the difficulty, when encountered, was corrected by cleaning and replating the platinum wire with mercury. Then 1 ml of 0.1 mM hemoglobin was added as its carbon monoxide derivative and the solution was allowed to stand. The diffusion current fell gradually over a 5 minute period, then remained constant so that routinely the hemoglobin was allowed to stand 20 minutes with a slight excess of mercury before completing the titration, to allow for complete binding of the mercury by the hemoglobin. The titration was completed with further 0.1 ml increments of 1 mM mercuric chloride. With hemoglobin dialyzed free of glutathione, values between 5 and 6 mercury-combining groups per hemoglobin molecule were generally recorded, although values as low as 4 were determined on some hemoglobin solutions seemingly titrated in an identical manner.

RESULTS

Studies on the mechanism of oxidation of glutathione

Since the sulfhydryl group of glutathione can be measured by simple and reliable photometric procedures, this thiol provides a convenient substrate for study of the mechanism of action of oxidant drugs. However, in working with glutathione its known property of being readily auto-oxidized must be considered. As auto-oxidation occurs much more rapidly at high pH and temperature (11) these variables were strictly controlled. Trace amounts of metal ions which catalyze the oxidation of GSH were presumably present even in resin "deionized" water and were chelated when desired by dilute ethylenediamine tetraacetate (EDTA). GSH was stable at room temperature in 1.5 per cent metaphosphoric acid in the period of 1 to 2 hours it took to analyze the solutions.

1. Oxidation of glutathione by phenylhydrazine and acetylphenylhydrazine with oxygen. Phenylhydrazine and acetylphenylhydrazine require oxygen to oxidize GSH, whereas ferricyanide does not, as can be seen in Table I. In addition, this table illustrates the effect on acetylphenylhydrazine of preincubation with oxygen. The acetylphenylhydrazine, dissolved in an oxygen-free deionized buffer containing no EDTA, was preincubated in Erlenmeyer flasks sealed with vaccine caps for 3 hours, either under nitrogen or under oxygen (room air). Acetylphenylhydrazine incubated under oxygen turned yellow, whereas that under nitrogen remained colorless. Next, the solutions of acetylphenylhydrazine were washed with nitrogen. Then glutathione, dissolved under nitrogen, was added with a syringe, and the solutions were incubated 0.5 hour longer with or without oxygen. It can be seen that acetylphenylhydrazine, if preincubated under oxygen, washed free of oxygen with nitrogen, and incubated with GSH under nitrogen, resulted in approximately the same destruction of GSH as when the oxygen was present during the incubation period as well.

Table II illustrates a similar experiment which shows that the auto-oxidation of both GSH and acetylphenylhydrazine is catalyzed by trace metal ions. Trace metal ions accelerated the oxidation of acetylphenylhydrazine by oxygen during preincubation as they did the subsequent oxidation of glutathione by the oxidized drug during incubation.

2. Hemoglobin and trace metals as catalysts for the oxidation of glutathione. The experiments illustrated in Figures 1 through 4 show the similarity between hemoglobin and trace metal ions in catalyzing the destruction of glutathione by oxi-

dant drugs. All experiments were performed in isotonic phosphate buffer, pH 7.4, and incubated under air at 37° C in 50 ml siliconized Erlenmeyer flasks in a Dubnoff metabolic shaker. In the experiments illustrated in Figures 2 and 4 trace metal ions were chelated by the presence of 0.01 per cent EDTA in all solutions used. The crystallized hemoglobin employed was present in 1 mM concentration, the acetylphenylhydrazine in 10 mM concentration, and the phenylhydrazine in 2 mM concentration. The reaction was stopped at the end of the incubation period by immersion of the flasks in ice water, aliquots being immediately removed and precipitated with 3 vol of 3 per cent metaphosphoric acid saturated with sodium chloride for measurement of total and reduced glutathione.

In these four figures the percentage of the initial reduced glutathione remaining is plotted against time. Although selection of concentrations of phenylhydrazine and acetylphenylhydrazine was arbitrary, additional experiments with different concentrations over different time periods confirmed the sequence of events to be described.

TABLE II

Effect of EDTA (0.01%) on oxidation of acetylphenylhydrazine (0.5%) during preincubation, as shown by its subsequent oxidation of GSH (40 mg %) in air in isotonic phosphate buffer, pH 7.4, at 37° C

Additions during preincub. (3 hrs)	Additions during incub. (0.5 hr)	% Glutathione after incub.
0	EDTA+GSH	100
0	GSH	80
0	APH+EDTA+GSH	94
0	APH+GSH	65
APH+EDTA	GSH	80
APH	EDTA+GSH	46
APH	GSH	33

Figure 1 illustrates the rapid oxidation of reduced glutathione, at 37° C and pH 7.4, with those trace metal ions present in resin deionized water. Glutathione alone was 40 to 50 per cent oxidized in 1 hour, and completely oxidized by 4 hours. Although acetylphenylhydrazine had negligible additional effect, phenylhydrazine increased the rate

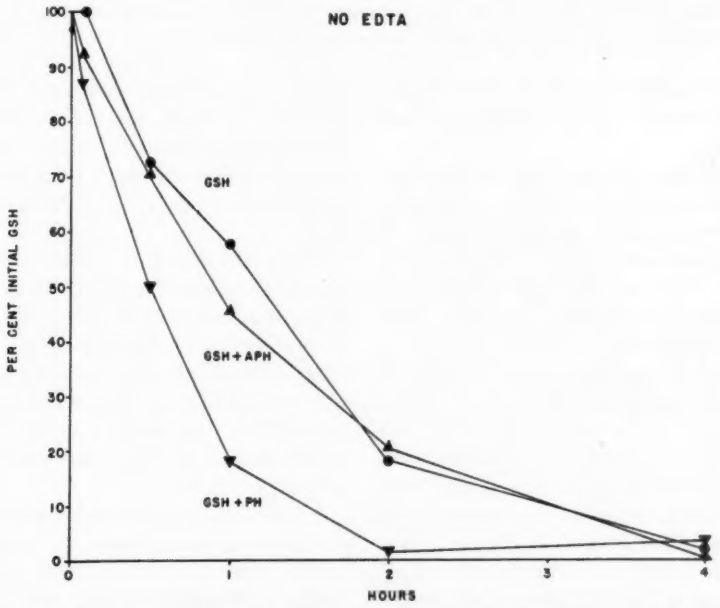

FIG. 1. THE OXIDATION OF REDUCED GLUTATHIONE (GSH) IN AIR, AT PH 7.4, 37° C, WITH AND WITHOUT ACETYLPHENYLHYDRAZINE AND PHENYLHYDRAZINE. The percentage of GSH remaining is plotted against the time of incubation for the control flasks ●, flasks with acetylphenylhydrazine ▲, and flasks with phenylhydrazine ▼. The solutions are made up in resin-deionized water but do not contain ethylenediamine tetraacetate (EDTA).

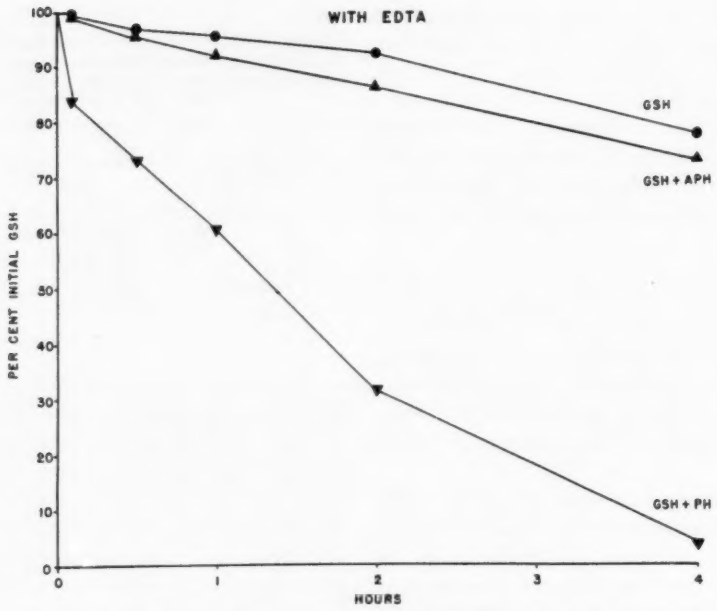

FIG. 2. THE OXIDATION OF GSH IN AIR, AT PH 7.4, 37° C, IN THE PRESENCE OF 0.01 PER CENT EDTA, EITHER ALONE ●, WITH ACETYLPHENYLHYDRAZINE ▲, OR WITH PHENYLHYDRAZINE ▼. The oxidation of GSH is slowed by chelation of trace metal ions, as can be seen by comparison with Figure 1.

of destruction, so that in its presence reduced glutathione was 50 per cent oxidized in 0.5 hour and gone by 2 hours.

Figure 2 illustrates the fact that reduced glutathione survives longer, even in the presence of acetylphenylhydrazine and phenylhydrazine, if metal ions have been chelated by EDTA. Again phenylhydrazine, but not acetylphenylhydrazine, increased the rate of destruction of GSH when compared with the control.

Figure 3 describes the time course of glutathione oxidation with hemoglobin but without EDTA. Hemoglobin alone, in the absence of EDTA, actually protected GSH (see below). The marked increase in the rate of GSH destruction with phenylhydrazine and acetylphenylhydrazine in the presence of hemoglobin, shown in Figure 3, was probably a result, therefore, of the capacity of hemoglobin to catalyze the oxidation of the drugs. Acetylphenylhydrazine, which without hemoglobin showed no demonstrable increased ability in this concentration to destroy glutathione, became markedly more active. Thus, in the presence of acetylphenylhydrazine and hemoglobin, 50 per cent oxidation of glutathione occurred in 0.7 hour as opposed to 1.2 hours for the glutathione alone, or 3.3 hours for glutathione and hemoglobin. Again, phenylhydrazine caused a more rapid destruction of glutathione, 50 per cent being oxidized in 0.3 hour.

Measurements of the total (reduced and oxidized) glutathione in the experiments illustrated in Figures 3 and 4 show that it decreased to 60 or 70 per cent of its initial level in the presence of either phenylhydrazine or acetylphenylhydrazine and hemoglobin, but did not change appreciably by itself or with hemoglobin during the 4 hour period studied, findings confirming previous work by others (5).

3. The sequence of changes with oxidant drugs, and with in vitro aging. Determination of the relative rates of GSH oxidation and of methemoglobin, sulfhemoglobin, and precipitate formation were most informative with acetylphenylhydrazine, since the changes were too rapid with phenylhydrazine for adequate study. Figure 5 shows a replot of the GSH measurements of Figure 4, this time as per cent conversion to oxidized glutathione

(GSSG) together with the percentage of the hemoglobin converted to methemoglobin, sulfhemoglobin or precipitate. Whereas both GSH oxidation to GSSG and hemoglobin oxidation to methemoglobin occurred early, no sulfhemoglobin or precipitated hemoglobin appeared until almost all GSH had been oxidized. However, the rate of GSH oxidation proceeded somewhat more rapidly than did methemoglobin formation, so that the chronological sequence was: *1*) GSH oxidation, *2*) methemoglobin formation, *3*) sulfhemoglobin formation and *4*) precipitation.

A similar, although more prolonged time course, is seen when glutathione is aged *in vitro* with hemoglobin and no oxidant drugs. Figure 6 illustrates an experiment in which 1 mM hemoglobin was incubated at 37° C under sterile conditions with 1 mM glutathione, at pH 7.4. It can be seen that, as in the presence of acetylphenylhydrazine, methemoglobin accumulated somewhat more slowly than did GSSG. As in Figure 3, GSH was 50 per cent oxidized in about 3 hours, whereas methemoglobin increased only slightly in this period. During the period of observation no sulfhemoglobin or precipitate formed, and hemoglobin sulfhydryls remained constant. The total glutathione level remained constant until after 24 hours, whereupon it began to decline and was half gone in 72 hours.

Analogous results are obtained if washed red cells from freshly defibrinated blood are incubated under sterile conditions in normal saline and glutathione levels are measured on the whole suspension. This was done and once again it was found that the oxidation of glutathione proceeded more rapidly than the formation of methemoglobin. Total glutathione decreased somewhat faster in the red cells (half-disappearance, 48 hours) than in a hemoglobin solution. In a parallel experiment the addition of 100 mg per 100 ml glucose to the initial isotonic saline delayed the entire process by approximately 24 hours.

4. Reactions between glutathione and hemoglobin derivatives. The interaction of various hemo-

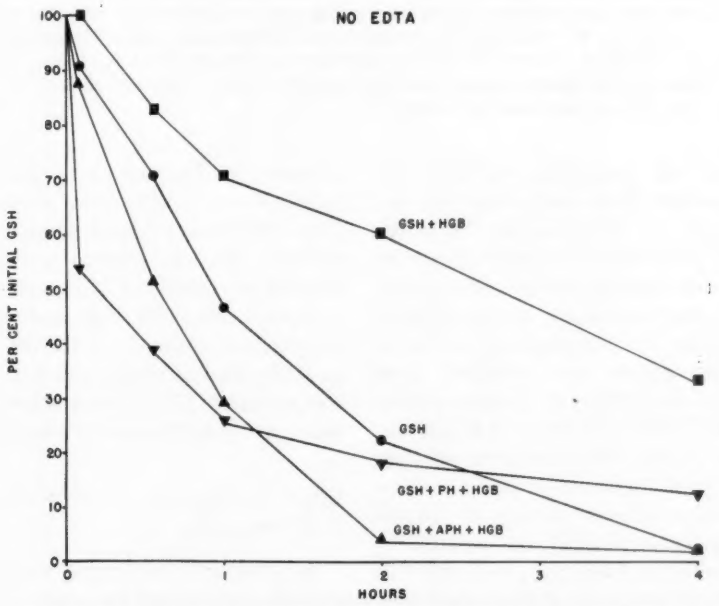

FIG. 3. THE OXIDATION OF GSH IN AIR, AT PH 7.4, 37° C, WITHOUT EDTA, BUT WITH EQUIMOLAR HEMOGLOBIN ■, WITH HEMOGLOBIN AND ACETYLPHENYLHYDRAZINE ▲, OR WITH HEMOGLOBIN AND PHENYLHYDRAZINE ▼, AS COMPARED WITH THE OXIDATION OF GSH ALONE ●. It can be seen that while hemoglobin alone protects GSH, hemoglobin catalyzed the oxidation of GSH by acetylphenylhydrazine and by phenylhydrazine (see Figures 1 and 2).

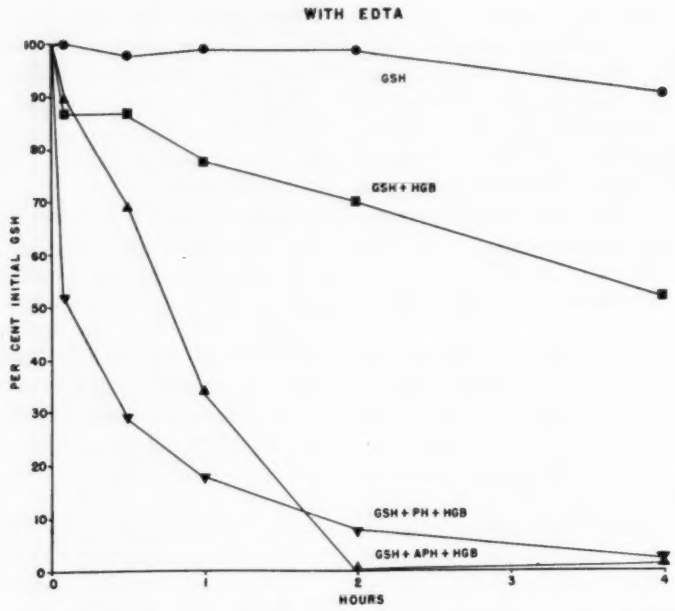

FIG. 4. THE OXIDATION OF GSH IN AIR, AT pH 7.4, 37° C, IN THE PRESENCE OF EDTA, EITHER ALONE ● OR WITH: EQUIMOLAR HEMOGLOBIN ■; HEMOGLOBIN AND ACETYLPHENYLHYDRAZINE ▲; OR HEMOGLOBIN AND PHENYLHYDRAZINE ▼. Note that the protective action of hemoglobin on GSH alone is now not in evidence with EDTA, and that the effect of EDTA in slowing the rate of phenylhydrazine and acetylphenylhydrazine oxidation of GSH has been circumvented by hemoglobin.

globin derivatives with glutathione was studied in experiments in which these derivatives were incubated with GSH or GSSG at pH 7.4 in the presence of 0.01 per cent EDTA at 37° in air for 0.5 hour. The short incubation time was intended to minimize the effect of oxygen, since removal of O_2 was not possible if oxyhemoglobin was to be studied. Methemoglobin was produced from oxyhemoglobin by the addition of 1.0 heme-equivalent of potassium ferricyanide followed by dialysis. Methemoglobin cyanide was produced similarly except that 1.0 heme-equivalent of neutralized sodium cyanide was added prior to dialysis. In this experiment 0.62 μmole of GSH was oxidized by each μmole of methemoglobin, compared with 0.21 by carbon monoxyhemoglobin, 0.15 by oxyhemoglobin, and 0.11 by methemoglobin cyanide. On the other hand, only 0.03 μmole or less of GSSG was reduced per μmole of each of the hemoglobin derivatives during this incubation period. Hence, methemoglobin leads to greater oxidation of glutathione than does oxyhemoglobin in air, although far less than a stoichiometric amount of sulfhydryl (4 μmoles) was oxidized during the time period studied. Since no reduced hemoglobin could be detected on reaction of GSH with methemoglobin, it appears that GSH was oxidized by molecular oxygen more efficiently in the presence of methemoglobin than of oxyhemoglobin. An inappreciable amount of GSSG was reduced by the 4 ferrohemes and 6 sulfhydryls of hemoglobin.

Effect of glutathione on the oxidative destruction of hemoglobin

In addition to the sequential relationship of glutathione and hemoglobin oxidation, as described in the previous section, evidence of a protective role of glutathione is provided by study of its inhibition of the oxidative destruction of hemoglobin.

The destructive effect of phenyhydrazine on hemoglobin in solutions containing equimolar re-

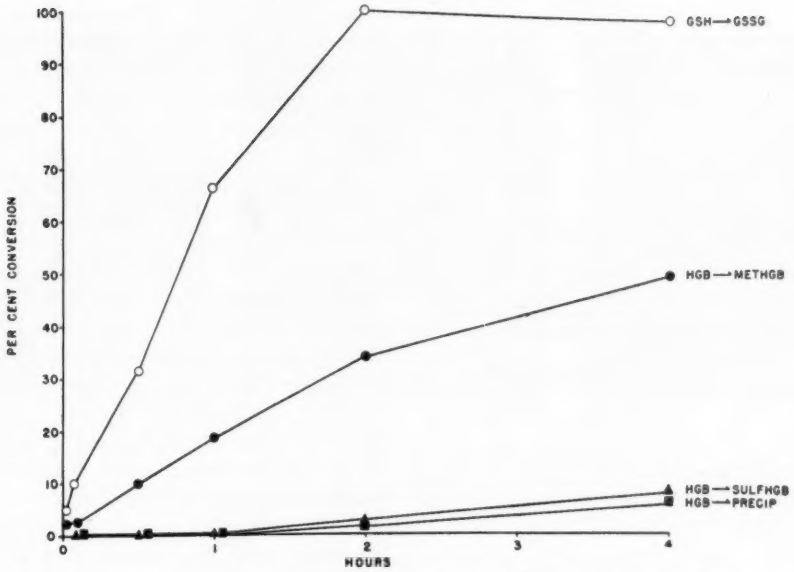

FIG. 5. COMPARISON OF THE RATES OF OXIDATION OF GSH AND OF EQUIMOLAR HEMOGLOBIN BY ACETYLPHENYLHYDRAZINE IN AIR. The data are from the same experiments as that illustrated in Figure 4. Note that GSH oxidation proceeds more rapidly than the conversion of hemoglobin to methemoglobin: GSH was half-oxidized in 45 minutes, hemoglobin half-oxidized in 4 hours. Further oxidative denaturation of hemoglobin (sulfhemoglobin formation and precipitation) occurred much more slowly.

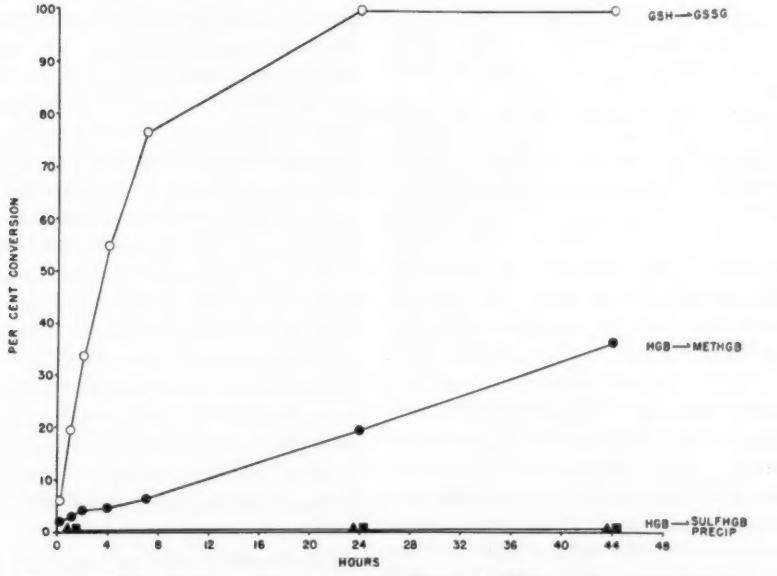

FIG. 6. COMPARISON OF THE RATES OF OXIDATION OF GSH AND OF EQUIMOLAR HEMOGLOBIN ON PROLONGED STERILE INCUBATION IN AIR AT 37° C. As in the presence of acetylphenylhydrazine (Figure 5), GSH oxidation proceeds more rapidly than methemoglobin formation. In this period of time virtually no sulfhemoglobin or precipitate formed.

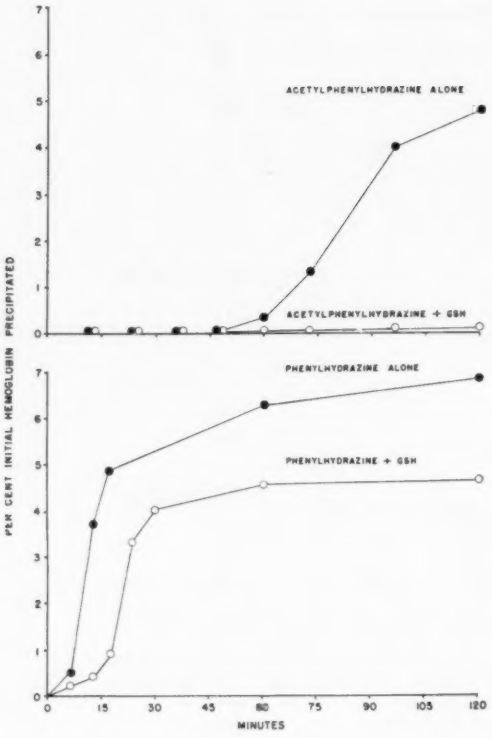

FIG. 7. EFFECT OF GSH ON THE PRECIPITATION OF HEMOGLOBIN BY ACETYLPHENYLHYDRAZINE (ABOVE) AND BY PHENYLHYDRAZINE (BELOW). At 6-minute intervals, half-millimolar amounts of GSH were added to the incubation mixture of 1 mM hemoglobin and 20 mM hydrazine derivative. A similar result was obtained when all of the GSH (10 mM) was added at the onset. Note the greater suppression of acetylphenylhydrazine than of phenylhydrazine.

duced glutathione[4] was inhibited, as compared with its effect on hemoglobin alone. Glutathione in such systems decreased the formation of methemoglobin by 30 to 40 per cent and, as shown in Figure 7, inhibited the rate of formation of precipitate. Larger amounts of glutathione produced greater inhibition but with a diminishing increment of effectiveness. GSH inhibited acetylphenylhydrazine more strikingly than it did phenylhydrazine. As indicated in Figure 7, the repeated addition of amounts of GSH (which were half the millimoles of hemoglobin present) completely blocked precipitation of hemoglobin by a 20 molar ratio of acetylphenylhydrazine. Methemoglobin formation was diminished, but not abolished, by GSH. Oxidized glutathione had no effect on the action of either hydrazine compound or hemoglobin.

In addition to inhibiting the quantity of hemoglobin precipitated by oxidant compounds, GSH, but not GSSG, caused two qualitative changes in the precipitate. Grossly, hemoglobin precipitated by phenylhydrazine in the presence of GSH or cysteine, formed coarse aggregrates of granules, as contrasted to the finely dispersed aggregates appearing in the absence of GSH. A second effect of GSH was to increase the size of the individual granules formed, as shown in Figure 8. Granules of hemoglobin precipitated by phenylhydrazine reach a diameter of from 1 to 2 μ. In the presence of GSH (but not of GSSG) such granules generally grew to a diameter of 2 to 3 μ, and sometimes to 4 μ. Cysteine, but not cystine, also had this effect. Whereas the effect of thiols such as GSH and cysteine on the gross aggregation of hemoglobin appeared to be unique, their effect on the microscopic size of the individual granules was apparently a function of the slower rate of granule formation. This interpretation, characteristic of crystal formation, was suggested by the finding that granules of hemoglobin precipitated by the slowly-active acetylphenylhydrazine were larger than those caused by the rapidly-active phenylhydrazine. Furthermore, granules formed within a short period by high concentrations of a given oxidant compound were smaller than those formed over long periods by low concentrations.

When solutions of crystallized hemoglobin were treated with GSH, dialyzed free of GSH, and then exposed to phenylhydrazine, no effect of the GSH-pretreatment was manifest. Suspension of phenylhydrazine-precipitated hemoglobin in solutions of GSH did not affect the appearance of the precipitate, but caused a slight increase in its weight. The same was true when thioglycollate or mercaptoethanol was added to the precipitate.

Effect of sulfhydryl-blocking agents on hemoglobin solutions and on red cells

1. Precipitation of hemoglobin. Most studies employed the organic mercurial *p*-chloromercuribenzoate, a compound having a strong primary

[4] The figure 68,000 was employed for the molecular weight of hemoglobin. Note that the normal concentration in red cells of hemoglobin is approximately 5 mM and that of glutathione is 2 to 3 mM.

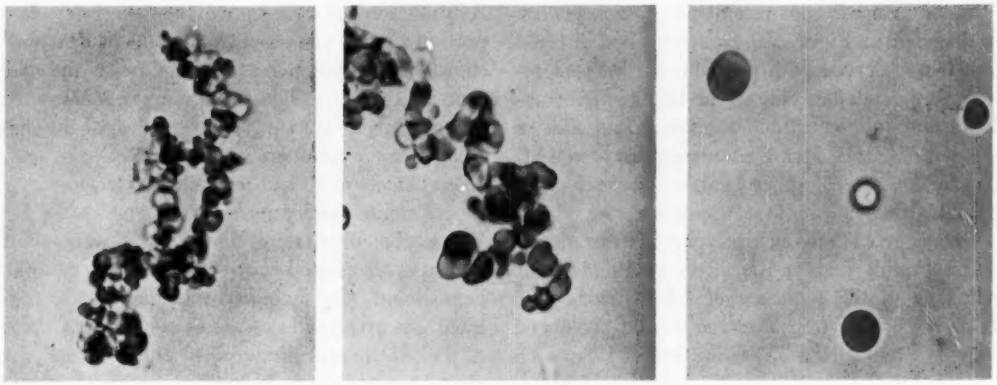

FIG. 8. EFFECT OF GSH ON THE SIZE OF THE GRANULES OF HEMOGLOBIN PRECIPITATED BY PHENYLHYDRAZINE. Phenylhydrazine alone (left) produced hemoglobin granules which grew to a diameter of 1 to 2 μ. The granules found when phenylhydrazine acted in the presence of GSH (middle and right) usually ranged in diameter up to 3 μ (middle) and occasionally to 4 μ (right). These variations in the size of granules of precipitated hemoglobin occurring without and with GSH are analogous to those encountered in GSH unstable red cells, and in normal red cells, respectively. Magnification × 2000.

affinity for sulfhydryl groups (11). The addition of PCMB to solutions of crystalline hemoglobin caused the gradual appearance of a red precipitate which consisted of granules that, apart from their color, were identical in appearance with those caused by oxidant compounds. This precipitation could be blocked stoichiometrically by GSH and did not occur at 0° C or under an atmosphere of carbon monoxide. GSH added to washed suspensions of hemoglobin precipitated by PCMB diminished the granule size but only partially solubilized the precipitate. As shown in the lower portion of Figure 9, up to 2 moles of PCMB per mole of hemoglobin caused essentially no precipitation of hemoglobin in 3 hours at 37° C. However, a molar ratio of PCMB in excess of 2 did cause precipitation to occur, beginning within 1 hour of admixture and proportional in amount to the dose of PCMB.

2. Heinz body formation and spherocytosis caused by PCMB. The ability of PCMB to hemolyze red cells has been well established by others (18). In the present studies, when to buffered suspensions of washed red cells equal amounts of 1 to 10 mM PCMB were added, the red cells were observed by microscopy to become spherical, to display progressively increasing osmotic fragility and later to hemolyze. Shortly prior to actual lysis, when the red cells manifested extreme osmotic fragility, fine marginal inclusion bodies, which stained dark blue with crystal violet, became visible. These inclusions became heavier as actual

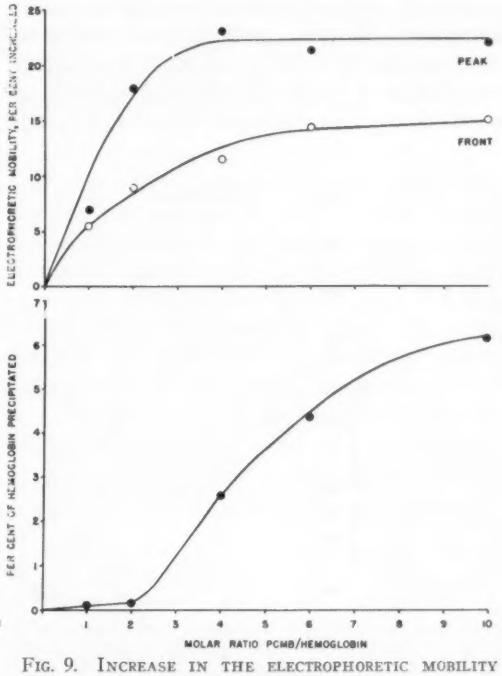

FIG. 9. INCREASE IN THE ELECTROPHORETIC MOBILITY (ABOVE) AND PRECIPITATION (BELOW) OF HEMOGLOBIN EXPOSED TO THE SULFHYDRYL-BLOCKING AGENT, *p*-CHLOROMERCURIBENZOATE (PCMB). Electrophoretic mobility in 0.05 M barbital buffer, pH 8.6, is expressed as the per cent increase over that of control (untreated) hemoglobin. Most of the increase in mobility, both of the main hemoglobin "peak" and of the fast-moving "front," was achieved with 2 moles of PCMB per mole of hemoglobin, whereas precipitation did not occur until more than 2 moles of PCMB had been added.

lysis commenced and resembled Heinz bodies. As depicted in Figure 10, this sequence differed from that observed with phenylhydrazine and related compounds not only in the lack of methemoglobin and sulfhemoglobin formation, but also in that, with PCMB, Heinz bodies appeared well after the onset of spherocytosis whereas with phenylhydrazine, the first appearance of Heinz bodies occurred long before spherocytosis developed. In this respect the effect of PCMB was analogous to the sequence of spherocytosis and Heinz body formation observed during prolonged storage of red cells at 37° C *in vitro* (1).

3. Changes in electrophoretic behavior of hemoglobin. Whether added to solutions of hemoglobin or to red cells, PCMB caused an increase in the electrophoretic mobility of hemoglobin on filter paper at pH 8.6. As shown in the upper portion of Figure 9, the increase in mobility of the main component at its color "peak" was evident on the addition of only 1 mole of PCMB per mole of hemoglobin and was almost maximally increased by the addition of 2 moles, so that only slight further increases appeared with up to 10 moles of PCMB. As shown in the same figure, the fastest detectable portion of the fast-moving component ("front") showed a similar increased mobility on the addition of PCMB. The fast component increased only slightly in amount at the expense of the main component. Another sulfhydryl-blocking agent, N-ethylmaleimide, had a similar effect.

4. Potentiation of phenylhydrazine by PCMB. When phenylhydrazine and PCMB were simultaneously incubated with solutions of crystalline hemoglobin, the amount of hemoglobin precipitated was greatly in excess of the combined weight of the precipitates produced by these agents when added separtely. As shown in Figure 11, the addition of 2 moles of phenylhydrazine per mole of hemoglobin caused less than 2 per cent of the hemoglobin to precipitate in a period of 2 hours in the absence of PCMB. The added presence of up to 2 moles of PCMB only slightly increased precipitation. However, with slightly larger amounts of PCMB, the degradation of hemoglobin was strikingly potentiated, with four to five times as much precipitate forming as after the addition of both of these compounds separately.

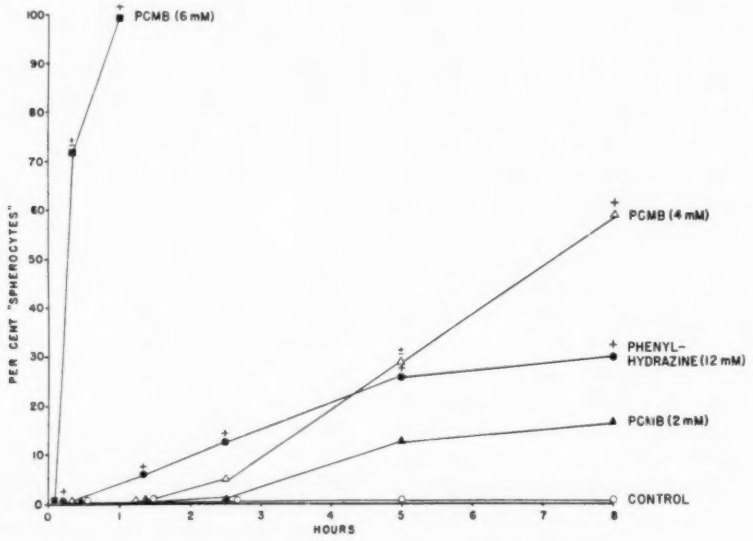

FIG. 10. SPHEROCYTOSIS AND HEINZ BODY FORMATION IN RED CELLS EXPOSED TO THE SULFHYDRYL-BLOCKING AGENT, *p*-CHLOROMERCURIBENZOATE (PCMB). Spherocytosis was quantitated arbitrarily by the per cent lysis in 0.50 per cent saline less the per cent lysis in 0.90 per cent saline (+ = presence of Heinz bodies). Note that while PCMB had a marked effect in causing sphering of red cells, Heinz bodies were a late event; with phenylhydrazine, however, the reverse was true.

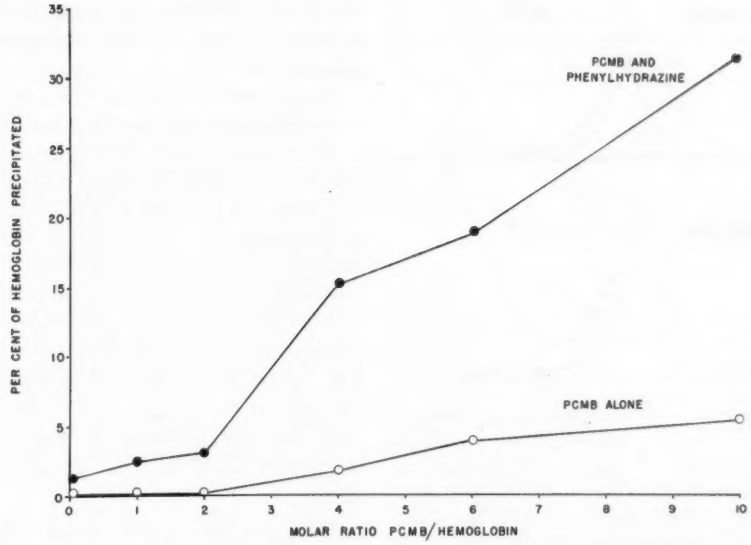

FIG. 11. POTENTIATION OF THE ACTION OF PHENYLHYDRAZINE ON HEMOGLOBIN BY THE SULFHYDRYL-BLOCKING AGENT, *p*-CHLOROMERCURIBENZOATE (PCMB). Although this dose of phenylhydrazine alone (●: first point) precipitated only about 2 per cent of hemoglobin, and as much as 10 moles of PCMB per mole of hemoglobin (○: last point) precipitated only 6 per cent, the combination of phenylhydrazine and 10 moles of PCMB precipitated 32 per cent of the hemoglobin.

This potentiating action of PCMB was blocked by the prior addition of equimolar amounts of GSH to the reaction mixtures.

Formation of hemoglobin-GSH mixed disulfides

The ability of hemoglobin to bind GSH is demonstrated in an experiment presented in Table III. When hemoglobin was incubated with ⅔ mole of $GS^{35}H$ per mole of hemoglobin at 37° C for 2 hours, a small but appreciable fraction (8.54 per cent) of the $GS^{35}H$ became precipitable by TCA along with hemoglobin, whereas virtually no $GS^{35}H$ was TCA-precipitable as the result of incubation with hemoglobin at 0° C. The presence of phenylhydrazine in the reaction mixture caused almost one-quarter of the $GS^{35}H$ (23.32 per cent) to attach to hemoglobin provided $GS^{35}H$ was present during the incubation period. However, if $GS^{35}H$ was added after the incubation period, but before TCA precipitation, there was no appreciable attachment of the label. That $GS^{35}H$ was largely bound to hemoglobin by disulfide linkage is suggested by the fact that about 80 per cent of the bound $GS^{35}H$ was removed from hemoglo-

TABLE III

Formation of hemoglobin-GSH mixed disulfides

Hemoglobin	Radioactive GSH: Added before incub.	Radioactive GSH: Added after 2 hrs incub.	Phenylhydrazine	Temp. ° C	% $GS^{35}H$ in TCA precipitate: Glycollic acid-treated	% $GS^{35}H$ in TCA precipitate: Thioglycollic acid-treated	% C^{14}-GSH in TCA precipitate: Glycollic acid-treated	% C^{14}-GSH in TCA precipitate: Thioglycollic acid-treated
+	+			0	0.18	0.29	0.22	0.37
+	+			37	8.54	1.54	3.96	1.44
+	+		+	37	23.32	5.11	17.40	5.25
+		+	+	37	1.37	0.27	1.42	0.73

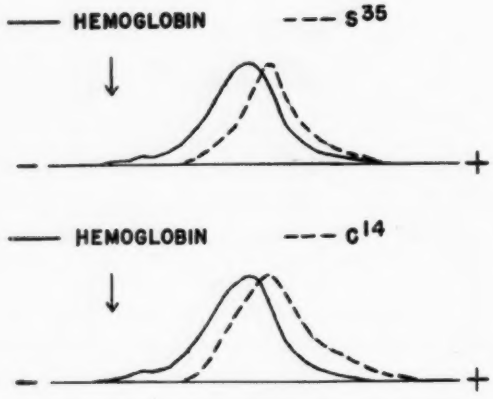

FIG. 12. ELECTROPHORETIC LOCALIZATION OF HEMOGLOBIN-BOUND GSH IN THE FAST COMPONENT OF HEMOGLOBIN. GSH labeled either with S^{35} (top) or C^{14} (bottom) became bound to hemoglobin during oxidation by phenylhydrazine. After dialysis, electrophoresis of the hemoglobin in 0.05 M barbital buffer, pH 8.6, followed by autoradiography, revealed that the GSH radioactivity (interrupted lines) localized in the faster-moving components of the hemoglobin (solid lines).

bin by thiollic reduction with thioglycollic acid, as compared with a control similarly treated with glycollic acid.

Studies with C^{14}-labeled GSH revealed a similar, albeit somewhat smaller, binding of C^{14} than of S^{35} to hemoglobin. This suggests that most of the bound material is intact glutathione but that a portion (as much as 25 per cent) of the hemoglobin-bound S^{35} may involve inorganic sulfur derived from the GSH preparation.

The specific activity of the portion of hemoglobin precipitated by phenylhydrazine in the presence of either $GS^{35}H$ or C^{14}-GSH was from 4 to 5 times that of the total hemoglobin (i.e., that precipitated by TCA). Thus, the most severely denatured (precipitated) hemoglobin had bound much more GSH than had the still-soluble hemoglobin.

After dialysis of the hemoglobin exposed to phenylhydrazine in the presence of labeled GSH, both $GS^{35}H$ and C^{14}-GSH moved with hemoglobin on filter paper electrophoresis in 0.05 M barbital

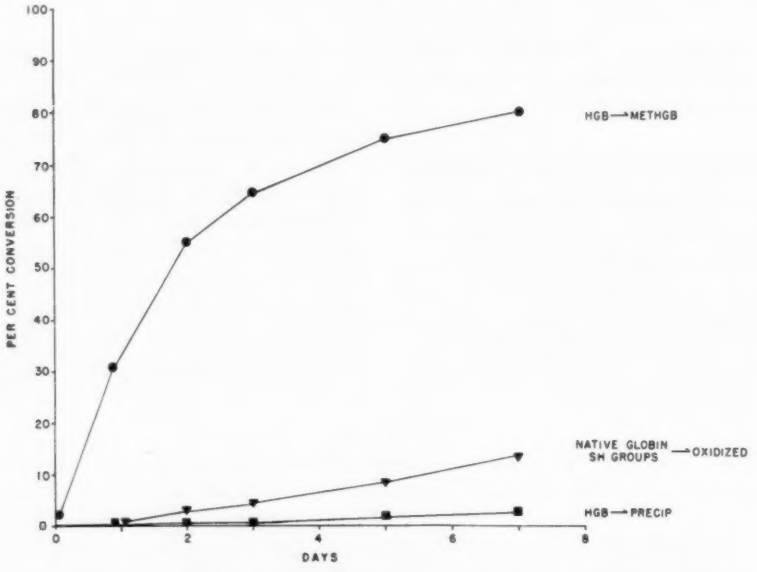

FIG. 13. COMPARISON OF METHEMOGLOBIN FORMATION, OXIDATION OF THE TWO SULFHYDRYL GROUPS OF NATIVE HEMOGLOBIN, AND PRECIPITATE FORMATION ON PROLONGED STERILE INCUBATION OF HEMOGLOBIN IN AIR AT 37° C. Note that methemoglobin formation comes first, then oxidation of native sulfhydryls which in turn precedes precipitation of hemoglobin. It will be recalled that when GSH was present in such a system, it was completely oxidized during the first day (Figure 6).

buffer, pH 8.6 (Figure 12). As this figure indicates, the radioactivity localized largely with the augmented fast-moving component of hemoglobin.

Measurement of hemoglobin mercury-binding sites

Our experience with mercuric chloride was similar to that of previous workers (19, 20) since a value of 2 mercury-binding groups per native (i.e., undenatured) hemoglobin molecule was obtained reproducibly. Measurements of total mercury-binding groups in 8 M urea frequently gave values less than the expected maximal figure of 6 (19) per hemoglobin molecule, due either to the uncertain stoichiometry of mercury, or to the lability of sulfhydryl groups under these conditions (20). Measurements of total sulfhydryls under experimental conditions here were made alternately with control hemoglobin samples, giving values of 5 to 6 mercuric ions per hemoglobin molecule.

Although previous workers have observed a fall in hemoglobin sulfhydryl groups on storage at 0° (19), no systematic study of this phenomenon has been undertaken by others. Figure 13 shows the results of amperometric titration of the mercury-binding groups of hemoglobin (undenatured by urea) which had been aged *in vitro* at 37° C for varying lengths of time. The percentage of the initial value of 2.04 mercury-binding groups per hemoglobin molecule is compared with percentage conversion to methemoglobin, and percentage precipitation. It is seen that oxidation of the heme groups (methemoglobin formation) precedes oxidation of the globin sulfhydryl groups, whereas the latter is synchronous with precipitation. In an additional series of experiments the precipitate formed from hemoglobin aged 8 days at 37° C was dissolved in 8 M urea, and its mercury-binding groups were compared with those in 8 M urea of the supernatant hemoglobin as well as of hemoglobin kept at 0° C for the same period of time. The results are shown in Table IV. It can be seen that the hemoglobin-derived precipitate has lost nearly all of its sulfhydryl groups. Not all forms of precipitation of hemoglobin result in loss of so many sulfhydryl groups. Thus, acetone precipitation of the supernatant hemoglobin incubated at 37° C or of the hemoglobin stored at 0° C gave values between 3 and 4 binding groups per hemoglobin molecule.

TABLE IV

Effect of incubation on the total mercury-binding groups of hemoglobin

Incubation conditions	Physical state of hemoglobin	Hemoglobin total mercury-binding groups titrated
0° C, 8 days	Soluble	5.8
37° C, 8 days	Partly soluble	
	a) Soluble supernate	5.7
	b) Precipitate	1.2

A similar decrease in sulfhydryl groups was found with hemoglobin treated with excess alkaline ferricyanide. In Table V it is seen that the supernatant hemoglobin from solutions treated with 14 moles of ferricyanide per mole of hemoglobin, at pH 9.2 for 1 hour, lost the 2 sulfhydryl groups available for mercury titration in the native protein, but retained 4 on titration in 8 M urea. The precipitate had lost almost all mercury-binding groups. Apparently, then, oxidation of the 2 outer sulfhydryl groups, during a short time period, is compatible with solubility, but further oxidation results in precipitation (1).

In order to study the effects of phenylhydrazine on the mercury-binding groups of hemoglobin 1 mM of hemoglobin was treated with 4 mM of phenylhydrazine for 4 hours at 37° C in phosphate buffer pH 7.4, then dialyzed overnight

TABLE V

Effect of ferricyanide on the mercury-binding groups of hemoglobin

Experimental conditions	Physical state of hemoglobin	Hemoglobin mercury-binding groups titrated	
		"Native"	Total
Untreated hemoglobin	Soluble	1.9	5.9
Alkaline ferricyanide-treated hemoglobin	Partly soluble		
	a) Soluble supernate	0	4.4
	b) Precipitate		0

TABLE VI

Effect of phenylhydrazine on the mercury-binding groups of hemoglobin

Experimental conditions	Physical state of hemoglobin	Hemoglobin mercury-binding groups titrated	
		"Native"	Total
Untreated hemoglobin	Soluble	1.9	5.9
Phenylhydrazine-treated hemoglobin	Partly soluble		
	a) Soluble supernate	1.7	5.6
	b) Precipitate		2.9

with mechanical stirring against 150 volumes of 0.85 per cent sodium chloride. As can be seen in Table VI, the number of mercury-binding groups of dialyzed, phenylhydrazine-treated hemoglobin is slightly lower than for untreated control hemoglobin, but considerably higher than for ferricyanide hemoglobin in which a like amount of precipitation had occurred. The mercury-binding groups of the hemoglobin precipitated by phenylhydrazine were diminished in number, but did not vanish as after precipitation by ferricyanide.

As A_I, the fast-moving component of hemoglobin on IRC 50 chromatography (13), is increased by *in vitro* aging and phenylhydrazine or alkaline ferricyanide treatment (1), it was of interest to determine the number of mercury-binding groups of this component. After chromatography of carbon monoxyhemoglobin in developer 2 (13) in which the cyanide had been replaced by an equivalent amount of sodium hydroxide, the components A_I and A_{II} showed no difference in the mercury-binding groups, either of the native proteins or after urea denaturation. Moreover, when the amount of the front-running component was increased by alkaline ferricyanide treatment from 10 to 17 per cent, the number of titrable sulfhydryl groups was decreased equally in both A_I and A_{II} hemoglobins, from 2.0 to 1.3.

DISCUSSION

Prevention of hemolysis and of the precipitation of hemoglobin as Heinz bodies in the normal red cell requires the suppression of chemical equilibrium with molecular oxygen. Heme ferrous iron and thiol groups of globin and glutathione exist in an oxygenated environment which, if equilibrium were attained, would rapidly result in their oxidation. The following representative values (21) of the oxidation potentials (Eo') of redox systems involving these substances (not all, it is true, for precisely the same conditions of pH and temperature) serve to indicate the relatively steep oxidative gradient found in the red cell: 0.2 atmosphere oxygen, 0.8v; hemoglobin $\rightleftarrows$ methemoglobin, 0.140v; GSH $\rightleftarrows$ GSSG, 0.04 v. The sulfhydryl groups of globin probably approximate the last two systems in oxidation potential, as may possibly the tyrosine and tryptophane residues of globin. Thus, if a redox equilibrium is permitted, electrons will flow from these red cell constituents to oxygen.

Fortunately for its survival, the red cell possesses many reducing systems which, as others have stressed (22), oppose the attainment of an intracellular redox equilibrium. Catalase destroys the reactive intermediate reduction product of oxygen—hydrogen peroxide (22). Methemoglobin reductase regenerates oxidized heme groups (23). Glutathione, which is synthesized (24) and kept reduced by complex enzyme systems (6), is present in fairly high concentrations and buffers hemoglobin and sulfhydryl-containing enzymes against oxidative destruction. As a result of these reducing enzyme systems in the red cell, the sequence of events during oxidative hemolysis may not proceed in the same order as would be anticipated from knowledge of the oxidation potential hierarchy defined above. Furthermore, oxidant compounds may possess special properties (as of charge or steric configuration) which may endow specificity of redox action. Ferricyanide, for example, oxidizes heme in preference to glutathione, at pH 7.4, whereas the converse occurs with oxygen alone or oxygen and phenylhydrazine and related compounds. Finally, congenital defects in specific reducing systems of the red cell result in the accumulation of different oxidized products. A deficiency of a methe-

moglobin reductase yields much methemoglobin but normal GSH by nitroprusside test (25). Such an accumulation of an oxidized product with a higher oxidation potential than other groups still reduced, does not invalidate the sequence to be described but merely demonstrates once again that the system is not normally in mobile equilibrium.

Biochemical sequence during oxidative hemolysis and precipitation of hemoglobin

However, despite this probability of variation, when hemoglobin solutions containing equimolar glutathione are exposed to oxygen, with or without oxidant drugs, a surprisingly regular sequence of events takes place, as has been described previously (1) and as Figures 6 and 7 further attest. The role of sulfhydryl groups in this series of events is the particular concern of the present discussion, because of their function in maintaining the integrity of red cell constituents.

The first step in the series of events leading to hemolysis by phenylhydrazine and related compounds appears to be the reaction of oxygen with these compounds to form oxidized derivatives. Evidence has been presented that this is catalyzed by hemoglobin as well as by trace metal ions. Next, the oxidized drug reacts with glutathione, ferrous hemes, and hemoglobin sulfhydryl groups, apparently in this order of decreasing reaction rate. It is likely that all of these reactions proceed simultaneously but that, because of their different reaction rates, those irreversible changes leading to sulfhemoglobin formation and hemoglobin precipitation are not apparent until glutathione has been completely oxidized.

1. Glutathione oxidation. It is well established that at a physiologic pH, auto-oxidation of thiols occurs relatively rapidly unless "all traces of metals are removed, e.g., with a chelating agent" (11), a fact manifest in the present studies (Tables I and II). A report to the contrary (5) may be due to the use of unbuffered systems, since, as discussed earlier, thiols are relatively stable at an acid pH such as that of unbuffered GSH.

Hemoglobin added to GSH was shown to have two opposite roles: one, when alone, in protecting GSH from oxidation; and the second, in the presence of oxidant drugs, in facilitating its destruction. When trace metal ions were removed by EDTA, as seen in Figure 4, the hemoglobin no longer was protective. Thus, GSH with EDTA present was more stable without than with hemoglobin. This suggests that the protective effect of hemoglobin when alone is a nonspecific metal-binding property similar to that of other proteins, albeit less efficient in this respect than EDTA.

The second role of hemoglobin as catalyst of GSH destruction with or without oxidant drugs can be studied by comparison of Figure 4 (hemoglobin as catalyst) and Figure 1 (trace metals as catalyst) with Figure 2 (control). Hemoglobin accelerates GSH destruction by oxidant drugs even more rapidly than do trace metal ions: there is a 5 minute half-life for GSH with hemoglobin and phenylhydrazine (Figure 4) versus a 30 minute half-life for GSH with trace metals and phenylhydrazine (Figure 1); and a 45 minute half-life of GSH with hemoglobin and acetylphenylhydrazine (Figure 4) as opposed to a 1 hour half-life with trace metals and acetylphenylhydrazine (Figure 1). That hemoglobin was more active than trace metals in facilitating destruction of GSH by oxidant compounds in these experiments may be due to its relatively greater concentration.

These findings differ in several respects from similarly-devised experiments previously reported by Beutler, Robson and Buttenwieser (5). These workers found that neither hemoglobin nor acetylphenylhydrazine alone caused oxidation of GSH, whereas hemoglobin either simultaneously or preliminarily incubated with acetylphenylhydrazine did oxidize GSH. They concluded that acetylphenylhydrazine did not per se destroy GSH but did so by producing an altered form of hemoglobin. The present studies, on the contrary, indicate that acetylphenylhydrazine alone oxidizes GSH once it has been converted under oxygen to an active form (Table I); this conversion is catalyzed by trace metals (Table II) or by hemoglobin (1, 26), but its action on GSH does not require trace metals (Table II). In a very comparable manner hemoglobin alone catalyzes GSH oxidation by oxygen and is also capable of forming oxidized derivatives which directly oxidize GSH. It is apparent that, unless controlled throughout, the buffering, metal-binding, and oxidase properties of hemoglobin may have conflicting experimental consequences.

In red cells incubated under sterile conditions *in vitro* the oxidation of GSH followed by a fall in total glutathione is similar to that observed previously by other workers (*27*).

2. Heme oxidation. Proceeding at a slightly slower rate is the oxidation of the ferroheme groups of hemoglobin to form methemoglobin. Both phenylhydrazine and acetylphenylhydrazine are reducing agents, capable of reducing not only methemoglobin but also ferricyanide. Thus, it must be the oxidized products of these drugs that remove electrons from the ferrous iron hemoglobin (*26, 28, 29*). It is of interest that in artificial mixtures, the relative proportions of glutathione and ferrous heme oxidized by acetylphenylhydrazine and oxygen (Figure 5) roughly correspond to what one would predict from their oxidation potentials listed above. Hence, it is likely that if an oxidant drug is present (Figure 5) or if sufficient time for incubation is allowed, when the specific reducing systems normally present in the red cell are removed ferroheme and glutathione are actually in equilibrium. However, this equilibrium is extremely sluggish *in vitro* as methemoglobin will oxidize only a small portion of GSH in 30 minutes, during which none of the methemoglobin is reduced. The previous report by others (30) of the reduction of methemoglobin by glutathione attests this slowness, the reduction of 0.7 equivalents of heme requiring 24 hours at room temperature even under carbon monoxide, which by binding the reduced hemoglobin favors the reaction as well as decreasing the auto-oxidation of GSH.

3. Globin oxidation. In view of the evidence obtained with PCMB, and with alkaline ferricyanide (1), that sulfhydryl groups are involved in the oxidative destruction of the hemoglobin molecule, a direct measure of these groups was undertaken. Of the available methods, amperometric titration using the mercuric ion has been successfully applied to undenatured hemoglobin by Ingram (19) and by Allison and Cecil (*20*) with identical results indicating the presence of 2.2 mercuric ions per mole of hemoglobin. The higher values of some workers using silver have been attributed to nonspecificity of binding of this cation (31). Results on hemoglobin denatured with sodium dodecyl sulfate with mercuric ion have not been consistent in the literature (19, 20), probably because the divalent mercury ion may combine with either one or two sulfhydryl groups in denatured hemoglobin.

Accompanying the oxidation of the ferroheme groups, but at a slower rate, the two sulfhydryl groups exposed in native hemoglobin were found to undergo oxidation. There is evidence that these two sulfhydryl groups play a vital role in hemoglobin function. Riggs (3) showed that the addition of two PCMB molecules per molecule of hemoglobin resulted in a similar change in the oxygen equilibrium, as had been observed previously with aged hemoglobin solutions *in vitro* (32). Wyman presented evidence that the sigmoid oxygen equilibrium curve is the result of a widespread (tertiary) configurational change in the hemoglobin molecule on oxygenation (33). The X-ray diffraction studies of Perutz and associates (34), demonstrated that these sulfhydryl groups occupy key positions where the two different chains of the hemoglobin half molecule are contiguous; and it is possible that these sulfhydryl groups exert their influence on the oxygen equilibrium curve by altering the rearrangement of the chains that occur on oxygenation. Recently, Riggs and Wells (35) have shown that these two reactive sulfhydryl groups are on the β chain of the hemoglobin, and have stressed the role of sulfhydryl groups in the Bohr effect; i.e., the influence of pH on the oxygen equilibrium curve (36).

With this evidence of the importance of these two sulfhydryl groups it is of considerable interest that, apart from the ferroheme groups, they bear the brunt of the initial oxidative assault on the hemoglobin molecule. Thus, when hemoglobin is titrated with increasing amounts of ferricyanide, after the four heme groups are oxidized two further groups are oxidized prior to precipitation. Amperometric titration of such ferricyanide-treated hemoglobin while still in solution shows that these two sulfhydryl groups are no longer available for mercury binding. Likewise, the total number of sulfhydryl groups measured in 8 M urea has been reduced approximately from six to four. A similar sequence is observed when PCMB is added to the hemoglobin, 2 moles per mole of hemoglobin being bound prior to any precipitation. Furthermore, if these two sulfhydryl groups are blocked with PCMB, phenylhydrazine

precipitation of the hemoglobin is greatly potentiated. Phenylhydrazine likewise produces a diminution in native globin sulfhydryl groups although of a much lesser degree. The possibility of binding of phenylhydrazine to hemoglobin cannot be ruled out. Since phenylhydrazine alone lowers the galvanometer reading, such hemoglobin-bound phenylhydrazine might give a falsely high value of mercury-binding groups. As shown in Figure 12, aging of hemoglobin at 37° C *in vitro* also leads to a decrease of titrable sulfhydryl groups in native globin. This decrease commences only after all of the glutathione has been oxidized and slightly anticipates and parallels hemoglobin precipitation.

Significance of "fast-moving" components of hemoglobin

Associated with oxidation of the first two sulfhydryls of globin is the occurrence in hemoglobin of changes in its oxygen dissociation curve (3) and, as shown here, in its electrophoretic mobility and its behavior on IRC 50 columns. However, it has not been shown that these changes necessarily represent identical alterations of the hemoglobin molecule. Riggs has found that the effects of PCMB and of aging on the oxygen dissociation curve of hemoglobin are reversible with GSH. However, such reversibility has not been observed with the changes in hemoglobin mobility on electrophoresis or on column chromatography. There is no difference in the number of titrable sulfhydryls between components A_I and A_{II} isolated by column chromatography whether in fresh hemoglobin or in hemoglobin in which the usual number of two sulfhydryl groups has been diminished by ferricyanide treatment. It seems likely, therefore, that A_I, the fast-moving component, is not simply the main component of hemoglobin with two sulfhydryl groups oxidized but rather a further oxidized or denatured product thereof. While evidence presented here suggests that oxidation of globin sulfhydryls usually follows methemoglobin formation, it is entirely possible that they may be oxidized first under some conditions such as by oxygen at a high pH (3).

The electrophoretic and chromatographic changes produced by oxidative denaturation of hemoglobin are of importance in the interpretation of hemoglobin abnormalities with mobilities similar to these fast-moving components. The reported (37) association of an unstable hemoglobin found in leukemia, with cresyl blue-staining inclusion bodies and increased fast-moving components on electrophoresis, is of especial interest. If this fast-moving component is indeed like those produced by oxidation (1) the likelihood of its being an acquired abnormality is increased. The instability of hemoglobin H (37), which consists entirely of β chains (38), and which therefore should contain four, instead of two, readily reactive –SH groups, may well be attributed to this added lability to oxygen.

The mechanism of hemoglobin precipitation

Hemoglobin precipitates very slowly under oxygen. Oxidation or blockade of more than two sulfhydryl groups in the presence of oxygen results in an accelerated precipitation of hemoglobin; thus, when more than 6 moles of ferricyanide or 2 moles of PCMB per mole of hemoglobin was added under oxygen, precipitation proceeded within an hour rather than several days. As with hemoglobin precipitated during prolonged incubation, hemoglobin precipitated by ferricyanide and dissolved in 8 M urea was found to have almost no titrable SH groups. The fact that, after the oxidation or blockade of two globin sulfhydryl groups, precipitation occurred only if oxygen was present suggests that interference with these two groups permitted subsequent auto-oxidation, probably irreversible, of other groups of the globin molecule with attendent denaturation. That this process occurs much more rapidly in the presence of redox intermediates such as phenylhydrazine is predictable in view of their known capacity to catalyze oxidation. Conversely, the marked effect of 2 or more moles of PCMB in rendering hemoglobin hypersusceptible to precipitation by phenylhydrazine and oxygen (Figure 11) indicates the protective function of globin sulfhydryls in terms of other oxidizable groups in globin, probably by maintaining the native molecular configuration.

The potentiating effect of a sulfhydryl inhibitor on hemoglobin precipitation by oxidant compounds is not to be confused with previous reports (4) of the ability of sulfhydryl inhibitors to convert normal red cells into "hypersusceptible" red cells in terms of Heinz body formation. The

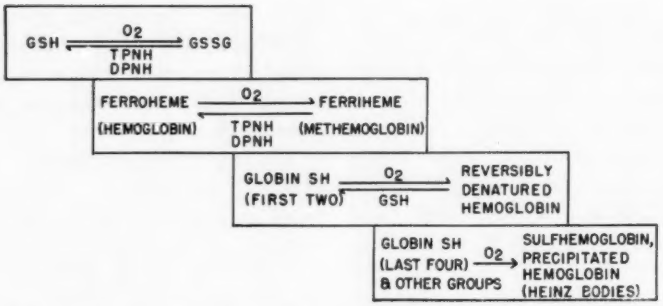

FIG. 14. SCHEME OF BIOCHEMICAL SEQUENCE DURING OXIDATIVE PRECIPITATION OF HEMOGLOBIN AND HEINZ BODY FORMATION.

latter effect was presumably due to injury to glycolytic enzymes (possibly glyceraldehyde-3-phosphate dehydrogenase), thereby inhibiting glycolysis.

Once precipitate has formed it cannot be redissolved by SH donors such as thioglycollate or mercaptoethanol. However, 8 M urea is effective in dissolving the precipitated hemoglobin. The effect of urea suggests hydrogen bonding between the precipitated hemoglobin molecules. Thus, while oxidation of the sulfhydryl groups disrupts the globular structure of the hemoglobin molecule so that precipitation occurs, probably hydrogen bonds, and not disulfide bonds, hold together the disrupted polypeptide chains of the precipitated molecules.

Figure 14 summarizes the overlapping series of reactions that occur during oxidative hemolysis. Within a given cell the levels of the oxidized products accumulated will be determined by the relative activity of the reducing mechanisms involved in each step.

Protection of hemoglobin by GSH and the formation of "fast-moving" mixed disulfides

That glutathione provides a primary defense against intracellular oxidation is well demonstrated in hemoglobin solutions *in vitro* by its ability to retard methemoglobin formation and inhibit hemoglobin precipitation (Heinz body formation) either by oxygen alone or by oxygen with oxidant drugs. In the red cell, glutathione is supported in this role by active reducing systems, since addition of glucose to the suspending medium likewise slows oxidative changes. The observations of Mills (6) indicate that in the intact cell, glutathione peroxidase increases the efficiency with which GSH serves as a reductant. It cannot be stated with certainty that the crystalline hemoglobin employed in the present studies was free from this enzyme; if the hemoglobin was comparable in purity to that of Mills, it is unlikely that glutathione peroxidase activity was involved. An interesting observation that explains the tendency of glutathione-deficient red cells to develop many small, rather than a few large, Heinz bodies (39) is that glutathione, by slowing the rate of precipitation, results in fewer but larger granules of precipitated hemoglobin.

In defending hemoglobin against oxidation, GSH is to some extent bound to hemoglobin. Thus, when either S^{35} or C^{14}-labeled GSH was mixed with both hemoglobin and phenylhydrazine, a portion of the GSH was precipitable with hemoglobin and traveled with hemoglobin during electrophoresis. That most of the labeled GSH could be detached with thioglycollic acid, but not by glycollic acid, confirms the supposition that this largely represents the formation of mixed disulfides during oxidation.

The appearance of labeled GSH in the fast-moving component of hemoglobin on electrophoresis suggests that the fast-moving hemoglobin of normal, senescent red cells may include hemoglobin-GSH disulfides and so represent another oxidative aspect of red cell aging *in vivo* (1). Indeed, there is unpublished evidence by Muller (40) that the fast-moving component of normal hemoglobin does contain glutathione. That the binding of GSH to hemoglobin by disulfide bonds during oxidation may account for the reported disappearance of GSH from red cells during hemolysis by primaquine and related phenylhydra-

zine-like drugs (4, 5) seems reasonable. On the other hand, there is no reason to doubt that some of the GSH is oxidized beyond the reversible, disulfide state and so becomes unmeasurable. In support of this, it was found that after exposure to phenylhydrazine alone there was a gradual decrease in the total recoverable glutathione.

The role of thiols in protecting against injury by ionizing radiations frequently has been attributed to their ability to inactivate free radicals created by these radiations (41). Eldjarn and Pihl (42), on the other hand, demonstrated the propensity of protective thiols such as cysteamine to form mixed disulfides with various proteins, including hemoglobin, both *in vitro* and in animals. These authors suggested that thiols exert a protective action by reversibly binding the protein sulfhydryl groups and preventing further, irreversible injury. The similarity of the mode of injury by radiation to that of oxidant drugs, and the similar protective roles played by thiols in these two situations, make this hypothesis equally plausible with respect to oxidative hemolysis.

A previous paper (1) called attention to the similarities between the sequence of events leading to Heinz body formation in red cells and the changes culminating in cataract formation in the lens of the eye. The present report confirms this parallelism. With both hemoglobin and lens crystallin (43) the sequence is, *1*) oxidation of glutathione, *2*) oxidation of protein sulfhydryl groups, and *3*) protein precipitation.

The fate of hemoglobin during oxidative damage as described in these studies is instructive in regard to possible mechanisms of cellular injury. Hemoglobin, being relatively rich in sulfhydryl groups and making up the bulk of the red cell protein, certainly is of chief importance in producing the morphological results of thiol oxidation, the Heinz bodies. However, it may be of no primary importance in determining red cell destruction. Glyceraldehyde-3-phosphate dehydrogenase, which is rich in sulfhydryl groups (44), and can be inhibited by –SH binding substances, may contain glutathione as a prosthetic group (45). This enzyme occupies a vital position in red cell glycolysis, and its inactivation may lead to an overwhelming of red cell reducing systems by oxidant drugs. Other sulfhydryl groups in other enzymes (46) and in the red cell membrane itself may well be involved in the cellular changes critical to the survival of the red cell *in vivo* (47). The observation here that sulfhydryl blockade by PCMB leads to a rapidly-ensuing spherocytosis and then hemolysis, occurring prior to the appearance of Heinz bodies, whereas spherocytosis and lysis are delayed manifestations of injury by phenylhydrazine and related compounds (1, 28), is consistent with the other evidence that such compounds must pass through an intermediate, hemecatalyzed step, prior to their critical action on cellular thiols, while the disruption of membrane integrity by PCMB is direct.

SUMMARY

During the oxidative injury of hemoglobin in solution or in red cells, thiol groups play a key role. Whether oxidation is spontaneous or accelerated by such electron-carrying compounds as phenylhydrazine, the following steps ensue.

1. Reduced glutathione (GSH) is oxidized to GSSG. In being oxidized a portion of the GSH becomes bound to hemoglobin by forming mixed disulfides with the globin sulfhydryl groups.

2. Hemoglobin is converted to methemoglobin as ferroheme becomes ferriheme.

3. The two reactive sulfhydryl groups of native hemoglobin are oxidized.

4. Oxidation of these two thiol groups probably results in the loss of the normal configuration of the hemoglobin molecule with the following consequences: *a*) the oxygen equilibrium (dissociation curve) is altered; *b*) fast-moving components of hemoglobin, including mixed disulfides of GSH and hemoglobin, appear on electrophoresis or chromatography; *c*) other reactive groups of the globin molecule, including the remaining four sulfhydryl groups, become susceptible to oxidation.

5. Brown to green hemochromes form ("sulfhemoglobin").

6. Denatured hemoglobin molecules polymerize, probably by hydrogen bonding, and precipitate to form coccoid granules (Heinz bodies).

Glutathione buffers hemoglobin against oxidation and may protect globin sulfhydryls by formation of mixed disulfides. As a result, GSH slows the precipitation of hemoglobin so that fewer, larger Heinz bodies are formed. Probably GSH

provides similar protection to other oxidatively labile constituents of the red cell such as the sulfhydryl groups of its enzymes and of its membrane. Presumably, injury to these latter components accounts for the actual hemolysis *in vivo* and for the spherocytosis observed *in vitro*.

ADDENDUM

Since the publication of the first paper in this series (1) it has come to our attention that E. L. Nelson had described the formation of spherical particles under certain conditions in water lysates of red cells (Nelson, E. L. The development in vitro of particles from cytoplasm. I. Observations on particle development in bacteriologic media. II. Particles from hemoglobin and desoxyribonucleic acid. J. exp. Med. 1958, **107**, 769). Of particular interest is the author's finding that at a low pH, particle formation could be induced in red cell lysates by the addition of desoxyribonucleic acid (DNA). Such particles contained a mixture of hemoglobin and DNA, and were capable during their formation of trapping and incorporating extraneous proteins and polysaccharides, which may explain the atypical staining characteristics of the inclusions of congenital inclusion body anemias.

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ERRATUM

In the paper of H. Bennhold and E. Kallee "Comparative studies on the half-life of I^{131}-labeled albumins and nonradioactive human serum albumin in a case of analbuminemia," J. clin. Invest. 1959, **38**, 863, "$CuSO_4$" in line 15 from the bottom of the right column of p. 864 should read "$CdSO_4$"; i.e., cadmium sulfate instead of copper sulfate.

EXTRATHYROIDAL ACTIONS OF PITUITARY THYROTROPIN: EFFECTS ON THE CARBOHYDRATE, LIPID AND RESPIRATORY METABOLISM OF RAT ADIPOSE TISSUE *

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The thesis that pituitary thyrotropin (thyroid-stimulating hormone, TSH) affects multiple facets of intrathyroidal metabolism and that changes in iodine economy may constitute merely one expression of these alterations (1, 2) is supported by many published reports. These are summarized in Table I. Only data derived by the direct addition of TSH to *in vitro* systems have been tabulated since, herein, the possible metabolic adaptations to changes in thyroid blood flow need not be considered.

The *in vitro* approach has demonstrated that a variety of the oxidative, assimilative and metabolic functions of surviving thyroid tissue are stimulated when TSH is introduced into media containing serum, or other potential organic substrates (3–8) (Table I, A). However, TSH also alters the respiration, hydration, and cytostructural lipids of thyroid tissue even in simple saline media (1, 9–12) (Table I, B). The occurrence of the latter phenomena in the absence of exogenous organic metabolites has prompted the suggestion that TSH *primarily* initiates a realignment of *existing* intrathyroidal pathways and/or a mobilization of *preformed* intrathyroidal substrates (5).

In order to find an extrathyroidal counterpart for this postulated sequence, the effects of TSH upon adipose tissue have been examined. The inquiry was stimulated by the suggestions of Engel, Engel and McPherson that the generalized metabolic action of pituitary hormones may mimic their effects upon target glands (13, 14). In the present studies it has been demonstrated that TSH effects net lipolysis in rat epididymal adipose tissue *in vitro* and that the lipolytic event is accompanied by augmented oxygen consumption and assimilation of glucose. Moreover, specific changes in the disposition of differentially-labeled glucose were effected. Since some of these phenomena could be reproduced by simply supplementing suspending media with albumin-complexed, long chain fatty acids, the experiments suggest that the mobilization of endogenous fatty acids may constitute one of the primary events in the TSH-induced changes in the oxidative and assimilative function of adipose tissue.

MATERIALS AND METHODS

1. Preparation of tissues and media. Epididymal adipose tissue was excised from rats weighing 200 to 280 g (Sprague-Dawley descendants obtained from Charles River Laboratories, Brookline, Mass.). Animals were allowed free access to food (Purina laboratory pellets) and water until the time of sacrifice by stunning, decapitation and exsanguination. Fat pads were exposed individually and divided in half so that a single donor rat was employed to fill four experimental vessels. Duplicate vessels were filled with proximal and distal halves of opposite fat pads in order to randomize anatomical-biological variation in paired control and experimental flasks. In a few studies, individual fat pads were cut into 3 segments so that 6 vessels could be filled with tissues from a single donor animal. Weights of incubated adipose segments ranged from 150 to 300 mg; for any single experiment, weights in individual flasks were adjusted to agree within ± 15 mg.

The following suspending media were employed. *a*) Modified Krebs-Ringer-bicarbonate (KRB): 0.120 M NaCl, 0.005 M KCl, 0.0012 M $MgSO_4$, 0.0008 M $CaCl_2$, 0.0012 M KH_2PO and 0.025 M $NaHCO_3$ in an atmosphere of 95 per cent O_2 − 5 per cent CO_2; or *b*) modified Krebs-Ringer-Tris-phosphate (KRT-P): 0.131 M NaCl, 0.005 M KCl, 0.0012 M $MgSO_4$, 0.0008 M $CaCl_2$ and 0.009 M Tris (hydroxymethyl) aminomethane buffer (pH 7.4) and 0.001 M sodium phosphate buffer (pH

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TABLE I

The effects of added thyroid-stimulating hormone (TSH) upon the intermediate metabolism of thyroid tissue in vitro

A. Effects in supplemented systems (i.e., incubation in serum or in saline media *supplemented* with organic substrates):

1. Increased consumption of oxygen (QO_2) within first hour (3–5).
2. Increased QO_2 and/or preservation of respiratory activity after prolonged incubation (4, 6).
3. Increased assimilation of glucose (5, 7).
4. Increased formation of $C^{14}O_2$ and lipid-C^{14} from glucose-U-C^{14} (5).
5. Formation of $C^{14}O_2$ from glucose-1-C^{14} greater than from glucose-6-C^{14} (7,8).
6. Increased incorporation of inositol-C^{14} into phosphoinositide (5).

B. Effects in nonsupplemented systems (i.e., incubation in simple saline media *without* added organic substrates):

1. Increased QO_2 within first hour (1).
2. Preservation of respiratory activity after prolonged incubation (9).
3. Preservation of water content during prolonged incubation (9, 10).
4. Increased incorporation of orthophosphate-P^{32} into phospholipids (1, 11, 12); sparing the "alkali-stable" phospholipids (12); and selectively affecting cephalins and phosphoinositide (1).

7.4) in an atmosphere of 100 per cent O_2. The KRT-P medium was employed to obtain a bicarbonate-free system containing physiological concentrations of inorganic phosphorus (3 mg per 100 ml) (15) and has been previously demonstrated to support the effects of TSH upon surviving thyroid preparations (1, 5). Enhancement of the glucose metabolism of adipose tissue by bicarbonate *in vitro* has been described by others (16, 17).

In some studies the media were modified to contain: *a*) unlabeled glucose (dextrose, National Bureau of Standards) ± D-glucose-1-C^{14} (lot 307D) or D-glucose-6-C^{14} (lot 341E, Volk Radiochemical Co.) to a final glucose concentration of 2 mg per ml ± 0.2 μc C^{14} per ml; *b*) dialyzed crystalline bovine albumin (Armour, lots V 68802 and V 68204) to a final albumin concentration of 20 mg per ml; or *c*) albumin-complexed sodium oleate (oleic acid, Nutritional Biochemicals Corp.) or sodium palmitate (palmitic acid, Fisher Scientific Co.) to a final concentration of 2 to 8 μmoles free fatty acids (FFA)[1] and 20 mg of dialyzed albumin per ml of suspending medium. In concentrations of 20 mg per ml, the albumin contributed 0.16 to 0.22 μmoles of titratable acid (presumably FFA) to each ml of suspending medium. Supplementation of the albumin with additional quantities of specific fatty acids was effected by a modification of the method of Bragdon and Gordon (20).

For studies of hormonal effects, the following preparations were employed.[2] *a*) ACTH: oxycellulose-adsorbed adrenocorticotropic hormone (ACTH, Wilson Laboratories, lot 104529, 130 USP units per mg; and lot 101900, 103 USP units per mg, from Dr. S. W. Hier). *b*) TSH: commercial "Thytropar" (Armour Laboratories, lots 2402 and U-3810, approximately 1 USP unit per mg; Armour Laboratories lot IRW, 1.5 to 2 USP units per mg, from Dr. S. Steelman); mouse tumor TSH [lot PC 11-113-B1, 7 USP units per mg, from Drs. R. W. Bates and P. G. Condliffe of Bethesda, Md. (21)]; beef TSH [lot PC 9-113 B3, "approximately 30 USP units per mg," from Drs. R. W. Bates and P. G. Condliffe (21)]; and beef thyrotropins, b + c [lot C-5-48-3, 15 to 20 USP units per mg, from Drs. J. G. Pierce and M. E. Carsten of Los Angeles, Calif. (22, 23)]. The beef preparations of Bates and Condliffe and Pierce and Carsten constitute the purest TSH that is currently available. The mouse tumor TSH is derived from transplanted pituitary tumors of athyreotic mice (21), a source in which the presence of other pituitary hormones should be minimal.

Lyophilized hormone preparations were weighed at the time of individual experiments with a Cahn electromagnetic balance, accurate to 1 μg, and dissolved in suspending media for addition to reaction vessels just prior to the start of incubation.

2. Incubation procedures. Incubation for 90 to 180 minutes at 38° C was performed in the Warburg apparatus (shaking rate, 104 cycles per minute) with conventional 15 ml Warburg vessels, or in the Dubnoff metabolic incubator (shaking rate, 90 to 100 cycles per minute) with rubber-stoppered Warburg vessels or 25 ml weighing bottles modified for gassing through needle vents. Two ml of suspending media was employed in the weighing bottles. In the Warburg vessels, 3.0 ml of suspending media was employed. These were placed directly in the main compartment or apportioned between

[1] The term free fatty acid, FFA, will be used in this manuscript as synonymous with nonesterified fatty acid, NEFA (18), and unesterified fatty acid, UFA (19).

[2] The author is greatly indebted to the various investigators whose generosity in sharing their limited supplies of highly purified hormone preparations made these studies possible.

the main compartment (2.7 ml) and side-arms (0.3 ml) for experiments involving timed "tip-in" of hormone. Details of gassing and manometry have been described elsewhere (24).

At the end of incubation, tissues were touched lightly to filter paper to remove adherent medium and were frozen on solid CO_2. Media were removed and processed directly or stored at −18° C for analysis on the following day. In experiments with glucose-C^{14}, processing of flasks at the end of incubation was modified to permit quantitative evolution of $C^{14}O_2$ into 0.5 M Hydroxide of Hyamine 10X [3] in methanol.

3. Analyses. In most experiments, final values were derived as the average of duplicate vessels. Although such replication minimized the number of possible observations from a single donor animal, it was felt that this was offset by the heightened accuracy thus afforded with an intrinsically variable biological preparation. In addition, all analyses of suspending media were performed with a minimum of two separate aliquots from each vessel.

For measurement of glucose concentration, protein-free filtrates of suspending solutions were prepared by precipitation with $Ba(OH)_2 - ZnSO_4$ and processed by the Nelson modification of the Somogyi method (25). Free fatty acids (FFA) in the media were estimated by the method of Dole (18), save for the substitution of Nile Blue A as the indicator solution. In a few instances (see below), the procedure of Gordon (26) was employed. Tissue FFA were measured by directly homogenizing the frozen segments in ground glass homogenizers with Dole extraction mixture and subsequent titration of heptane extracts as described by White and Engel (27).

For assay of $C^{14}O_2$, the alkaline Hyamine was quantitatively transferred into counting vials containing methanol : toluene (12 : 88 vol/vol) and 0.4 per cent 2,5-diphenyloxazole (PPO),[4] and 0.005 per cent 1,4-*bis* 2- (5-phenyloxazolyl)-benzene (POPOP).[4] Specimens were counted in an automatic Packard liquid scintillation counter.

4. Expression of results. Oxygen consumption was estimated in terms of microliters per hour per milligram of initial wet weight (QO_2). Net assimilation of glucose, expressed as micrograms per milligram of initial wet weight was calculated on the basis of the difference in the final glucose concentrations in vessels incubated with and without tissues, and a known volume of suspending medium within each vessel. Net changes in the FFA of the suspending media were similarly derived and expressed as micromoles FFA per gram of initial wet weight of tissue. Where tissue FFA were examined directly, values were related to the initial wet weight of the adipose segments. Oxidation of radioactive glucose into $C^{14}O_2$ was quantitated on the basis of recovered C^{14} and the specific activity of the glucose in the initial suspending medium. Results were expressed as micrograms of glucose-C^{14} per milligram of initial wet weight of tissue. In early experiments, attempts to use the weight of the dried "fat-free" residue of the adipose tissue as

[3] Purchased from Packard Instrument Company, Inc., La Grange, Ill.

[4] Purchased from Pilot Chemicals Co., Watertown, Mass.

TABLE II

The effect of thyroid-stimulating hormone (TSH) upon lipolysis in rat adipose tissue in vitro

Expt.* no.	Medium†			Time after "tip-in"‡	Final FFA				FFA $\frac{\text{tissue}}{\text{medium}}$ §	
					Medium		Tissue			
	Type	Alb.	Gluc.		−TSH	+TSH	−TSH	+TSH	−TSH	+TSH
				min	*μmoles/ml*		*μmoles/g*			
1	KRT–P	−	−	20	nd‖	nd	2.26	6.87	nd	nd
				60	nd	nd	2.33	7.22	nd	nd
				120	nd	nd	2.41	15.40	nd	nd
2	KRT–P	−	+	20	nd	nd	2.03	7.11	nd	nd
				120	nd	nd	1.76	7.53	nd	nd
3	KRT–P	+	−	150	0.41	1.04	3.75	10.58	9.1	10.2
4	KRT–P	+	+	150	0.30	0.97	3.05	9.22	10.2	9.5
5	KRT–P	+	+	20	0.25	0.31	2.25	9.24	9.0	29.8
				130	0.22	0.77	1.42	8.46	6.4	11.0
6	KRT–P	+	+	20	0.18	0.30	1.67	10.23	9.3	34.1
				120	0.17	0.81	1.29	11.10	7.6	13.7

* Each experiment number refers to studies with adipose tissue from a single rat. In experiments in which only single control and TSH values are listed (cf. 3 and 4), the data represent the averages of duplicate vessels.

† KRT–P refers to modified Krebs-Ringer-Tris-phosphate not supplemented (−) or supplemented (+) with 20 mg/ml of crystalline bovine albumin (Alb.) and/or 2 mg/ml of glucose (Gluc.). Main compartment of Warburg vessels filled with 2.7 ml of appropriate KRT–P and side-arms filled with 0.3 ml of same medium containing no TSH (−TSH) or 200 ug/ml (i.e., 1.4 USP units/ml) of Bates-Condliffe mouse tumor TSH (+TSH). Gas phase, 100% O_2.

‡ Side-arms "tipped" into main compartments following temperature-equilibration, gassing, and 40 to 60 minutes of control incubation. Final concentration of TSH in "+TSH" vessels was 20 μg/ml (i.e., 0.14 USP units/ml).

§ See text.

‖ None detectable.

TABLE III

The effects of various preparations of TSH upon the release of FFA into the suspending medium during incubation of rat adipose tissue

Medium *			Incubation		TSH†			No. of rats	FFA release	p
Type	Alb.	Gluc.	Volume	Duration	Type					
			ml	*hrs*		*μg/ml*	*U/ml*		*μmoles/g ± SE*	
KRB	+	−	2	1.5	0	0	0	9	2.00 ± 0.32	
			2	1.5	IRW	500	0.75–1.0	2	7.66	‡
			2	1.5	R2402	500	0.50	2	12.64	‡
			2	1.5	BC-beef	16	~0.50	2	6.05	‡
			2	1.5	Tumor	36	0.25	3	5.29§	‡
KRB	+	+	2	1.5	0	0	0	11	−0.02 ± 0.16	
			2	1.5	R2402	1,000	1.0	2	8.58	‡
			2	1.5	R2402	500	0.50	2	10.67	‡
			2	1.5	BC-beef	16	~0.50	2	4.68	‡
			2	1.5	Tumor	36	0.25	3	6.24§	‡
			2	1.5	R2402	100	0.10	2	9.67	‡
			2	1.5	Tumor	10	0.07	2	7.96	‡
KRB	+	+	3	3.0	0	0	0	2	−1.05	
			3	3.0	PC-beef	5	0.07–0.10	1	9.11	
			3	3.0	Tumor	5	0.035	1	7.25	
KRT-P	+	−	3	3.0	0	0	0	2	2.64	
			3	3.0	Tumor	20	0.14	2	12.07	
KRT-P	+	+	3	3.0	0	0	0	10	−0.14 ± 0.26	
			3	3.0	BC-beef	5	~0.17	1	8.80	‡
			3	3.0	Tumor	20	0.14	5	10.54 ± 0.78	<0.01
			3	3.0	PC-beef	5	0.07–0.10	1	7.59	‡
			3	3.0	Tumor	10	0.07	2	9.65	‡
			3	3.0	Tumor	5	0.035	1	7.58	‡

* As in Table II.

† IRW and R2402 refer to commercial preparation Thytropar (Armour) assaying 1.5 to 2.0 and 1.0 USP units/mg, respectively; 0 = no TSH. BC-beef = Bates-Condliffe purified beef TSH (PC 9-113B3) assaying "about 30 USP units/mg." PC-beef = Pierce-Carsten purified beef thyrotropins b + c (C-5-48-3) assaying "15–20 USP units/mg." Tumor = Bates-Condliffe mouse tumor TSH (PC 11-113-B1) assaying "7 USP units/mg." Lyophilized TSH preparations were dissolved in suspending media to obtain the concentrations in μg/ml and USP units/ml (U/ml) listed above and on subsequent tables.

‡ Differs from mean by more than two SD.

§ Analyses for FFA performed by the method of Gordon (26).

the reference standard for analytical results (i.e., by extracting each segment with 2:1 chloroform-methanol after incubation, or a representative segment prior to incubation) did not significantly enhance reproducibility or reduce animal-to-animal variation.

Statistical methods for assessing significance of differences between paired control and experimental vessels have been described in other publications from this laboratory (5, 28).

RESULTS

1. The effect of TSH upon the lipid metabolism of adipose tissue. As shown in Table II, the "tipping-in" of mouse tumor TSH was followed by a profound augmentation in the FFA pool of the incubated adipose tissue. Near-plateau values were effected within 20 minutes. The increased concentration of intracellular FFA occurred in albumin-free as well as in albumin-containing systems. However, whereas no FFA appeared in the medium in the absence of albumin, the presence of extracellular albumin was accompanied by a progressive release of FFA into the suspending medium. Since the absolute tissue content of FFA did not appreciably change during the 20 minute to 3 hour interval, the rising level of FFA within the medium presumably reflected continuing lipolysis in excess of the rate of FFA utilization by adipose tissue. Thus, during serial observations, tissue to medium concentration gradients for FFA, i.e., FFA [tissue (μmoles/g)/medium (μmoles/ml)], progressively declined and approached values observed in the control vessels (Table II).

The magnitude of concentration differentials for FFA between tissue and medium seemed to be

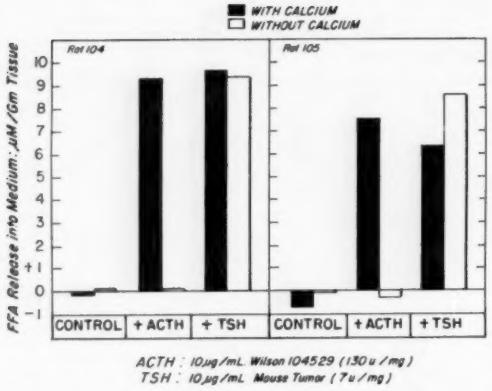

FIG. 1. THE INDUCTION OF LIPOLYSIS IN RAT ADIPOSE TISSUE BY ACTH AND TSH *IN VITRO*: THE RELATIONSHIP OF EXTRACELLULAR IONIC CALCIUM TO HORMONE ACTION. Concentrations of ACTH or TSH in the final reaction mixture are denoted by μg/ml. The potencies of the hormone preparations employed in these experiments are expressed in terms of USP units per mg (U/mg).

largely conditioned by the concentration of albumin in the suspending fluid [i.e., the abundance of extracellular acceptor sites for FFA (*29*)]. Accordingly, in subsequent documentation of the effects of various TSH preparations upon FFA, media containing 20 mg per ml of crystalline bovine albumin were employed, and lipolytic potencies were compared solely on the basis of change in the FFA concentration of the medium. Results of experiments with five different preparations of TSH in concentrations of 0.01 to 1.0 USP unit TSH per ml are summarized in Table III. In KRB as well as KRT-P, all preparations of TSH effected a release of FFA into the suspending medium which exceeded by at least threefold the modest FFA production occasionally observed in paired control vessels.

Despite the positive findings with highly purified TSH preparations, the possibility still remained that the stimulation of lipolysis was induced by trace contamination with other pituitary principles. Of the adenohypophyseal factors that are known to activate lipolysis, only ACTH has sufficient *in vitro* activity on a weight basis to qualify as such a contaminant. ACTH was excluded by the technique of Lopez, White and Engel (30) (Figure 1). Six segments of epididymal adipose tissue from single donor rats were incubated individually for 1 hour in Ca^{++}-free KRB containing 1 mg per ml glucose, 20 mg per ml albumin, and 1 mg per ml EDTA.[5] Thereafter, tissues were lightly blotted and introduced into similar Ca^{++}-free or Ca^{++}-containing KRB solutions which had been further supplemented with nothing (control), or 10 μg per ml Oxycel ACTH (ACTH), or 10 μg per ml mouse tumor TSH (TSH). Two of four such experiments are pictured in Figure 1. In confirmation of Lopez and associates, the activity of ACTH was not manifest in the absence of extracellular ionic calcium. To the contrary, the activity of TSH was unaffected.

2. The effects of TSH upon the respiratory and carbohydrate metabolism of adipose tissue. The introduction of TSH (Figure 2) effected a prompt augmentation of QO_2 that was manifest at the earliest manometric readings (i.e., 10 to 20 minutes) following "tip-in." Concomitantly, the assimilation of glucose from the suspending medium was increased. Results of experiments performed with five different preparations of TSH in concentrations exceeding 0.030 USP unit per ml are shown in Table IV. Respiratory enhancement was invariable. It occurred in albumin-free as well as in albumin-containing KRT-P and did not require glucose for initiation. However, in both systems, the presence of glucose not only enhanced QO_2 in control flasks, but also caused a greater and more sustained stimulation of oxygen consumption by TSH. Effects upon glucose assimilation were more variable, presumably, in part, because of the analytical limitations of deriving values on the basis of minor changes in the suspending medium. Nonetheless, for every type of medium, the effects of TSH in concentrations of 0.03 to 1.0 USP unit per ml (Table IV and the 10 μg per ml values from Table V) were highly significant (p, 0.01 or less). To assess whether some parallelism existed between the effects of TSH upon glucose and respiratory metabolism, a rank order correlation coefficient (31) was derived for the 25 experiments with KRT-P in which both parameters were measured concurrently. The correlation between the absolute increase in QO_2 (ΔQO_2) and the absolute change in the assimilation of glucose (Δ glucose assimila-

[5] Ethylenediamine tetraacetic acid dihydrate.

tion) proved to be of marginal statistical significance (R, 0.470; p, $< 0.05 > 0.01$).

For glucose and QO_2, as for the release of FFA, no clear dose-response relationship could be delineated when individual animals which had been examined at a single TSH concentration in the 0.03 to 1.0 USP unit per ml range were intercompared (Tables III and IV). Therefore, experiments were performed with smaller quantities of TSH. Results of five experiments are shown in Table V. As little as 1 μg per ml (0.007 USP unit per ml) of mouse tumor TSH elicited detectable augmentation of lipolysis, glucose assimilation, and oxygen consumption. Moreover, when paired adipose segments from a single donor animal were compared, the effects of 1 μg per ml upon all parameters were smaller in magnitude than the changes that were induced by 10 μg per ml. In the latter experiments, the fourth piece of adipose tissue was incubated concurrently with

TABLE IV

The effect of various preparations of TSH upon oxygen consumption and glucose assimilation by rat adipose tissue in vitro

Expt.* no.	Medium†			TSH‡			QO_2			Glucose assimilation		
	Type	Alb.	Gluc.	Type			−TSH	+TSH		−TSH	+TSH	
					μg/ml	U/ml	μl/mg/hr		Δ	μg/mg		Δ
7*	KRB	+	+	R2402	1,000	1.0				1.29	1.69	0.40
8*		+	+	R2402	1,000	1.0				2.24	1.94	−0.30
9*		+	+	BC-beef	16	0.5				0.15	1.12	0.97
10*		+	+	BC-beef	16	0.5				0.21	0.98	0.77
11*		+	+	Tumor	36	0.25				0.13	0.42	0.29
12*		+	+	Tumor	36	0.25				0.60	0.59	−0.01
13*		+	+	Tumor	36	0.25				0.34	0.81	0.47
14*		+	+	PC-beef	5	0.07–0.10				1.73	3.04	1.31
15*		+	+	Tumor	10	0.07				0.58	1.38	0.80
16*		+	+	Tumor	10	0.07				0.64	0.65	0.01
17*		+	+	Tumor	5	0.035				2.47	3.25	0.78
18	KRT–P	−	−	U 3810	330	0.33	0.105	0.139	0.034			
		−	+				0.118	0.224	0.106	0.36	1.39	1.03
19		−	−	U 3810	330	0.33	0.112	0.188	0.076			
		−	+				0.130	0.243	0.113	0.25	1.16	0.91
20		−	+	Tumor	50	0.35	0.166	0.271	0.105	0.92	1.57	0.65
21		−	−	Tumor	20	0.13	0.126	0.154	0.028			
		−	+				0.131	0.203	0.072	0.34	0.56	0.22
22		−	−	Tumor	20	0.14	0.141	0.164	0.023			
		−	+				0.169	0.210	0.041	0.76	0.41	−0.35
23		−	+	Tumor	10	0.07	0.112	0.174	0.062	0.51	1.31	0.80
24		−	+	Tumor	10	0.07	0.077	0.148	0.071	0.70	1.14	0.44
25		−	+	Tumor	10	0.07	0.147	0.172	0.025	0.52	0.96	0.44
26		−	+	Tumor	10	0.07	0.171	0.239	0.068	0.66	0.79	0.13
27		−	+	Tumor	10	0.07	0.090	0.140	0.050	0.30	0.99	0.69
28		−	+	Tumor	10	0.07	0.117	0.138	0.021	1.41	0.66	−0.75
29		−	+	Tumor	10	0.07	0.127	0.220	0.093	1.14	1.39	0.25
30	KRT–P	+	+	BC-beef	5	0.15	0.127	0.178	0.051	0.49	1.27	0.78
31		+	+	Tumor	20	0.14	0.200	0.265	0.065	0.92	0.76	−0.16
32		+	−	Tumor	20	0.14	0.210	0.278	0.068			
		+	+				0.361	0.519	0.158	1.69	3.50	1.81
33		+	−	Tumor	20	0.14	0.183	0.192	0.009			
		+	+				0.214	0.263	0.049	1.09	0.82	−0.27
34*		+	+	Tumor	20	0.14	0.263	0.319	0.056			
35*		+	+	Tumor	20	0.14	0.155	0.253	0.098			
36*		+	+	PC-beef	5	0.07–0.10	0.123	0.183	0.060	0.52	1.42	0.90
37		+	+	Tumor	10	0.07	0.139	0.151	0.012	0.69	1.20	0.51
38		+	+	Tumor	10	0.07	0.200	0.242	0.042	1.52	1.78	0.26
39		+	+	Tumor	10	0.07	0.097	0.140	0.043	0.46	1.32	0.86
40*		+	+	Tumor	5	0.035	0.123	0.170	0.047	0.49	0.78	0.29

* Experiments in which media were also assayed for FFA.

† KRB refers to modified Krebs-Ringer-bicarbonate and KRT-P to modified Krebs-Ringer-Tris phosphate not supplemented (−) or supplemented (+) with albumin (Alb.) and/or glucose (Gluc.), as in Table II. In all KRB studies except 14 and 17, volume of suspending medium was 2.0 ml and incubation was for 90 minutes at 38° C. In experiments 14 and 17, and all KRT-P studies, suspending medium consisted of 3.0 ml and incubation was for 3 hours. Gas phase, KRB, 95% O_2–5% CO_2; KRT–P, 100% O_2.

‡ TSH, as in Table III. U 3810 represents commercial preparation Thytropar (Armour) assaying 1 USP unit/mg; μg/ml and U/ml (USP units/ml) refer to final concentration in reaction mixture.

TABLE V

The parallelism between the effects of TSH upon lipid, respiratory and carbohydrate metabolism of rat adipose tissue in vitro

Expt. no.	Medium* Type	Alb.	Gluc.	Mouse tumor TSH	Release FFA into medium	QO_2	Glucose assimilation
				μg/ml	μmoles/g	μL./mg/hr	μg/mg
41	KRT–P	+	+	0	−0.14	0.195	1.00
				1.0	3.08	0.231	1.72
42	KRT–P	+	+	0	−0.32	0.127	0.56
				10.0	10.30 [−0.76]†	0.219 [0.127]	1.64 [0.85]
				1.0	3.48	0.176	1.24
43	KRT–P	+	+	0	−1.16	0.281	2.14
				10.0	9.01 [0.26]	0.368 [0.254]	2.46 [1.82]
				1.0	3.55	0.296	2.27
44	KRT–P	−	+	0	nd	0.139	0.88
				10.0	nd	0.199 [0.173]	1.71 [0.77]
				1.0	nd	0.185	1.35
45	KRT–P	−	+	0	nd	0.126	0.65
				10.0	nd	0.212 [0.151]	1.62 [1.22]
				1.0	nd	0.156	1.15

* As in Table II. The μg/ml of TSH refers to the final concentration of Bates-Condliffe mouse tumor TSH (7 U/mg) in the 3.0 ml reaction mixture. Incubation, 3 hours.

† Values in brackets denote results obtained with adipose tissue from the same animals when 10 μg/ml of TSH (Bates-Condliffe mouse tumor) which had been heated at 96° to 98° C for 3 minutes at pH 2 was employed; nd = none detectable.

10 μg per ml of mouse tumor TSH which had been heated (96° to 98° C) for 3 minutes at pH 2. The results of such mild acid-heating upon the lipolytic, respiratory and assimilative potencies of TSH are shown by the values in brackets in Table V. In every instance, the activities of TSH upon lipolysis, oxygen consumption, and glucose assimilation were reduced at least 90 per cent. When comparable experiments were performed with ACTH (Figure 3), no measurable inactivation was produced by mild acid-heating, thus further indicating that the effects of TSH upon lipid, respiratory and carbohydrate metabolism did not result from ACTH contamination.[6]

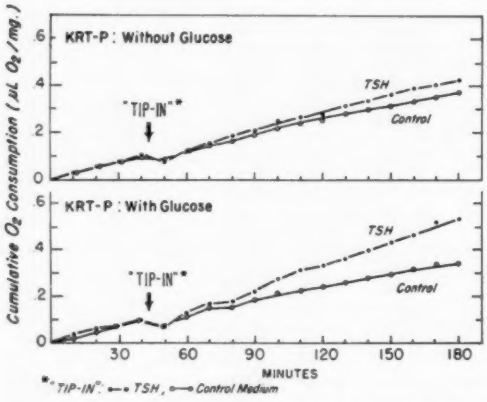

FIG. 2. THE EFFECTS OF TSH UPON THE OXYGEN CONSUMPTION OF RAT ADIPOSE TISSUE: INFLUENCE OF EXTRACELLULAR GLUCOSE. Adipose segments from a single donor animal were employed to fill four vessels. Paired vessels were incubated in KRT-P with or without 2 mg glucose per ml. After control observations, mouse tumor TSH in sufficient amounts to yield 20 μg per ml was tipped into one of each pair of vessels. Comparable quantities of KRT-P without added hormone were tipped into the control flasks.

The actions of TSH upon the differential oxidation of C-1- and C-6-labeled glucose were assessed in five experiments with the three most purified preparations of hormone (Table VI). In KRT-P as well as KRB media, TSH effected minimal changes in the oxidation of glucose-1-C^{14}. However, a two- to sixfold increase in the evolution of $C^{14}O_2$ from glucose-6-C^{14} was produced. As a consequence of the altered disposition of assimilated glucose, the average C-1/C-6 ratio for evolved $C^{14}O_2$ declined from 3.9 in control vessels to 1.2 in the presence of TSH.

3. The effects of free fatty acids upon the respiratory and carbohydrate metabolism of adipose tissue. To evaluate the possibility that some of the effects of TSH upon respiratory and carbohydrate metabolism might result from the increased intracellular availability of FFA, paired segments of adipose tissue were incubated *a*) in

[6] The use of mild acid-heating as a means of differentiating between TSH and ACTH by selective inactivation was suggested by Dr. Robert W. Bates.

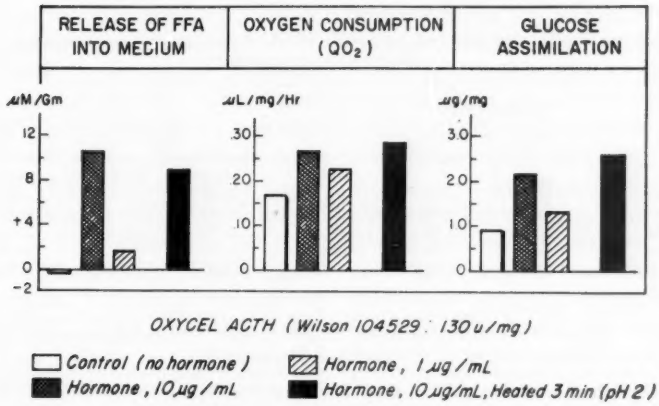

FIG. 3. DOSE-RESPONSE RELATIONSHIPS IN THE ACTIONS OF ACTH UPON ADIPOSE TISSUE: EFFECTS OF MILD ACID-HEATING UPON ACTH POTENCY. Adipose segments from a single donor animal were employed to fill four vessels containing respectively: a) KRT-P plus glucose, 2 mg/ml, plus albumin, 20 mg/ml (control); *b*) as in *a* but supplemented with 10 μg/ml ACTH; *c*) as in *a* but supplemented with 1 μg/ml ACTH; *d*) as in *a* but supplemented with 10 μg/ml of ACTH which had been heated (96° to 98° C) for 3 minutes at pH 2.

control systems with media containing glucose, albumin and 0.18 to 0.22 μmole per ml of titratable fatty acids, and *b*) in experimental systems in which the FFA of these media had been supplemented with oleate or palmitate. As judged by final tissue/medium concentration differentials for FFA (Table VII), the addition of FFA to the suspending medium resulted in a true increase in the intracellular pool of FFA (i.e., tissue/medium ratios exceeded unity). Moreover, in confirmation of the findings of others (32–34), cellular utilization of the FFA was demonstrated by failure to recover all of the added palmitate or oleate. In association with this disposition of FFA, the oxygen consumption of surviving adipose segments was uniformly enhanced (Figure 4). Mean stimulation of QO_2 above control values averaged 33 ± 6 per cent [7] in 13 experiments. Effects of the added oleate or palmitate upon the differential oxidation of C-1- and C-6-labeled glucose were investigated in nine experiments (Table VIII). The oxidation of glucose C-1 was increased slightly in eight of the nine studies, whereas the formation of $C^{14}O_2$ from glucose-6-C^{14} was augmented in every instance, and, proportionally, to a greater extent. Results in KRB and KRT-P systems were qualitatively similar.

[7] Mean ± standard error of the mean.

TABLE VI

The effect of various preparations of TSH upon the oxidation of glucose-1-C^{14} and glucose-6-C^{14} by rat adipose tissue

Expt. no.*	Medium*	TSH*	Oxidation of glucose carbon to CO_2					
			C-1		C-6		C-1/C-6	
			−TSH	+TSH	−TSH	+TSH	−TSH	+TSH
			μg C^{14}-glucose/g initial wet weight					
14	KRB	PC-beef	556	575	143	856	3.9	0.7
17	KRB	Tumor	967	919	207	543	4.7	1.7
30	KRT–P	BC-beef	141	142	36	207	3.9	0.7
36	KRT–P	PC-beef	152	268	42	181	3.6	1.5
40	KRT–P	Tumor	96	139	26	91	3.7	1.5

* Numbers refer to the same experiments as in Table IV. All media contained glucose (2 mg/ml) and crystalline bovine albumin (20 mg/ml) and either were (+TSH) or were not (−TSH) supplemented with 5μg/ml of TSH as above.

TABLE VII

The disposition of albumin-bound free fatty acids (FFA) during incubation of rat adipose tissue in glucose-containing media

Expt. no.	Medium FFA* Type	Initial	Final	Δ	Tissue FFA Final		Net† Δ	FFA tissue/medium
				μmoles	*μmoles*	*μmoles/g*	*μmoles*	
46	Oleate	1.78	1.35	− 1.29	0.40	2.43	−0.89	1.8
47	Oleate	8.10	3.84	−12.78	6.54	30.37	−6.24	7.9
48	Oleate	8.28	3.94	−13.02	6.03	29.48	−6.99	7.5
49	Oleate	8.35	3.94	−13.23	9.70	46.13	−3.53	11.7
53	Palmitate	1.62	1.22	− 1.20	0.30	1.50	−0.90	1.2

* Medium consisted of 3 ml KRT–P containing glucose (2 mg/ml), crystalline bovine albumin (20 mg/ml), and FFA complexed to the albumin in concentration designated as "initial" above. Concentration of titratable fatty acids in the albumin prior to addition of palmitate or oleate was 0.18 to 0.22 μmoles/ml.

† Net Δ (μmoles): minimal estimate of the net quantity of FFA which disappeared from the system during 3 hours of incubation at 38° C and in an atmosphere of 100% O_2. The value was derived as the difference between the change in the FFA of the suspending medium (i.e., medium Δ; [Initial-Final] × 3), and the tissue FFA (μmoles) at the end of incubation. Corrections for alterations in the endogenous FFA of the tissue during incubation were not made.

Thus, C-1/C-6 ratios for $C^{14}O_2$ evolution were invariably reduced by the enrichment of suspending media with naturally occurring free fatty acids.

DISCUSSION

The present studies indicate that thyrotropin must be added to the growing list of pituitary principles (17, 27, 35–43) which can exert a direct influence upon the metabolism of surviving rat adipose tissue. At least superficially, it appears that the *in vitro* response to several of these hormones is quite similar. Thus, with TSH, as with ACTH (27, 39, 43) and growth hormone (STH) (27), lipolysis is activated *in vitro*. Like ACTH (37, 42, 43) and STH (17, 36), TSH also promotes a selective increase in the oxidation of the sixth carbon of glucose. Finally, TSH enhances the net assimilation of glucose, and even in the absence of glucose, stimulates the oxygen consumption of adipose tissue. During the preparation of this manuscript, comparable properties have been reported for ACTH and for a purified bovine growth hormone preparation (41, 43). Whether the seeming uniformity of response reflects contamination of the available ACTH, TSH and STH preparations with one another or even with other pituitary components cannot be answered with complete finality. For growth hormone, the contaminant possibility is rendered tenable by the large quantities that are required for manifest action *in vitro* (17, 27, 36, 41). However, in the case of ACTH and TSH, the hormone requirements are of much smaller magnitude. Moreover, fairly effective dissociation of ACTH and TSH can be achieved. In the present studies it has been demonstrated that a degree of mild acid-heating, which does not affect ACTH, diminishes the activity of TSH upon all of these aspects of adipose tissue metabolism and that the lipolytic potency of TSH, unlike that of ACTH (30), is not contingent upon the avail-

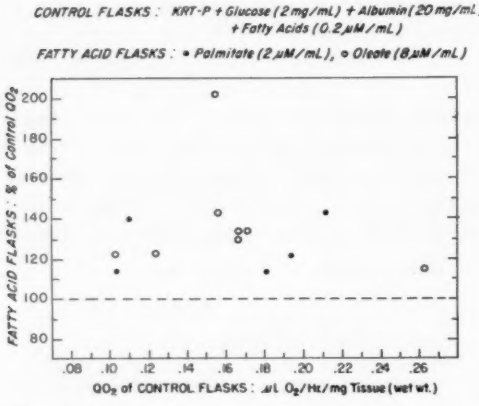

FIG. 4. EFFECTS OF NATURALLY OCCURRING FATTY ACIDS UPON THE OXYGEN CONSUMPTION OF RAT ADIPOSE TISSUE. "Fatty acid flasks" denote vessels in which control media have been further supplemented with palmitate (2 μmoles/ml) or oleate (8 μmoles/ml). Values for oxygen consumption by adipose tissue in the enriched systems have been expressed as a percentage of the QO_2 which was observed in the paired control flasks. By this convention, 100 per cent (i.e., the dotted line) denotes no difference between control and experimental flasks.

ability of extracellular ionic calcium. In addition, corroborative experiments were performed with the highly purified beef thyrotropins of Bates and Condliffe, and Pierce and Carsten. Their isolation by sequential cationic and anionic exchange chromatography should exclude more basic components, such as ACTH, and minimize the inclusion of more acidic products (21–23). Furthermore, in the TSH derived from mouse pituitary tumors, one might anticipate negligible contamination with other pituitary hormones even in the initial starting material (21). Thus, the likelihood that the above effects of TSH can be ascribed to any known pituitary principles besides TSH would seem to be vanishingly small. Nonetheless, until the precise structure of all adenohypophyseal hormones has been elucidated, the possibility cannot be excluded that the seemingly similar intracellular activities of ACTH, TSH and perhaps STH in adipose tissue are mediated by a common peptide component [in the manner of the pigment effects of α-MSH and β-ACTH (44)] and that apparent differentiations by selective inactivations affect only those configurational characteristics of the intact hormone that govern transcellular transport.

The multiple effects of TSH upon the carbohydrate, lipid and respiratory metabolism of the epididymal fat pad prompted some effort to distinguish between primary and secondary events. The possibility that the increased intracellular availability of FFA might be implicated was subjected to preliminary examination. Instead of augmenting FFA from within by inducing lipolysis with TSH, the absolute quantities of both esterified and unesterified intracellular fatty acids were increased from without by adding oleate or palmitate to suspending media containing albumin and glucose. This resulted in a reduplication of the increased oxygen consumption and the selective alteration in the oxidation of differentially labeled glucose.[8] Concurrently, Cahill, Leboeuf and Flinn reported similar effects of added palmitic acids upon the oxidation of glucose (49) and Lynn has shown that added butyrate stimulates "glucose uptake, respiration and fat synthesis from glucose" in adipose tissue (50). The precise mechanism by which changes in carbohydrate and respiratory economy are effected by fatty acids remains obscure and whether *every* aspect of TSH action in adipose tissue can be reproduced in this fashion must await more detailed chemical dissection. However, if the latter

[8] Parenthetically, it should be noted, that in some of these experiments, concentrations of added fatty acids were employed that might obtain during periods of lipid mobilization *in vivo* (45). The ensuing alterations in the disposition of glucose may have intriguing implications for the pathogenesis of some of the complications which are observed in such clinical states as diabetes mellitus, wherein chronic, intermittent elevations of plasma FFA occur (46). In the least, however, the findings that FFA can influence the carbohydrate metabolism of isolated adipose tissue warrant consideration in the use of the epididymal fat pad for bioassay of insulin-like activity (47), and may, conceivably, be involved in some of the anomalous results with plasma from totally pancreatectomized animals (48).

TABLE VIII

The effect of high concentrations of albumin-bound FFA upon the oxidation of glucose-1-C^{14} and glucose-6-C^{14} by rat adipose tissue

Expt. no.	Medium*	FFA* Type	FFA*	Oxidation of glucose carbon to CO_2: C-1 −FFA	C-1 +FFA	C-6 −FFA	C-6 +FFA	C-1/C-6 −FFA	C-1/C-6 +FFA
			μmoles/ml	*μg C^{14}-glucose/g initial wet weight*					
48	KRT–P	Oleate	8.28	209	433	87	234	2.4	1.8
49	KRT–P	Oleate	8.35	105	121	70	127	1.5	0.9
50	KRT–P	Oleate	8.04	46	58	17	33	2.7	1.8
51	KRT–P	Oleate	8.12	149	151	78	153	1.9	1.0
52	KRB	Oleate	6.06	471	633	146	354	3.2	1.8
53	KRT–P	Palmitate	1.62	136	167	53	86	2.6	1.9
54	KRT–P	Palmitate	2.20	250	350	38	130	6.6	2.7
55	KRB	Palmitate	1.74	362	264	110	132	3.3	2.0
56	KRB	Palmitate	1.74	253	274	110	174	2.3	1.6

* Numbers refer to the same experiments as in Table VII. All media contained glucose (2 mg/ml) and crystalline bovine albumin (20 mg/ml). The albumin either was (+FFA) or was not (−FFA) complexed with added fatty acids as above. Concentration of titratable fatty acids in the −FFA media was 0.18 to 0.22 μmoles/ml.

obtains, as seems quite likely, then the potentiation of TSH action upon adipose tissue QO_2 which was observed in the presence of glucose suggests that a supporting role must be assigned to other processes directly or indirectly concerned with the disposition of glucose.

Although many other isolated tissues have been examined (1, 7), the fat pad seems to be the only preparation in which an extrathyroidal action of TSH can be demonstrated. From the available data, it is hard to assess whether the effects of TSH upon adipose tissue *in vitro* are of physiological significance *in vivo*. The lower limits of responsiveness of adipose tissue were not explored but changes were routinely elicited with concentrations of TSH that are only five to ten times greater than the upper levels of TSH in normal human plasma (21). In the intact animal, the possibility that TSH conjointly activates the thyroid and mobilizes oxidizable extrathyroidal metabolites in response to increased peripheral metabolic demands has undeniable teleological attraction. Similarly, it would be tempting to ascribe the exophthalmogenic potential of certain thyrotropins to extrathyroidal actions upon the metabolism of retro-ocular lipids [or other components (51)]. However, the mouse tumor TSH which was employed for some of the present experiments has no exophthalmogenic activity (52), and many of the previous observations of generalized metabolic alterations following the administration of TSH (14, 53) were obtained in animals with intact thyroid glands. In view of the prompt rise in plasma FFA after the exhibition of thyroid hormone (54), it appears that athyreotic animals should be employed for assessment of the adipokinetic potential of TSH *in vivo*. Such animals should also receive adequate replacement therapy on the conceivable basis that TSH, like epinephrine (55), may require permissive quantities of thyroid hormone for maximal activation of lipolysis *in vivo*. In this regard, the observations of Hetzel, Charnock and Good may be germane (56). Two of their five human subjects with treated myxedema responded to injected TSH with acute elevations of oxygen consumption although they dismissed the findings as "due to non-specific stress." Re-examination of the entire problem of extrathyroidal TSH actions seems warranted. Of special interest might be investigation of adipose tissue metabolism in mice with thyrotropin-secreting tumors of the pituitary, since plasma TSH levels are chronically elevated in these animals (21).

Motivating all of the present efforts was the hope that clues might evolve relevant to the author's sustaining interest in the mechanism of action of TSH within the thyroid. On the basis of previous studies, we had postulated that the primary intrathyroidal action of TSH is independent of organic extracellular substrates but that concomitant or secondary changes in thyroid assimilative function are necessary for sustained TSH action (5). Insofar as TSH within the fat pad: *a*) stimulates oxygen uptake in simple saline media; *b*) promotes glucose assimilation in glucose-containing media; and *c*) enhances QO_2 maximally in the presence of extracellular glucose, an extrathyroidal counterpart for the proposed sequence of TSH action within the thyroid has been obtained. Moreover, insofar as TSH activates lipolysis in adipose tissue, a model system has been described wherein intracellular substrate can be augmented by TSH independent of extracellular supply.[9] Indeed, the fact that some of the effects of TSH upon oxygen consumption and glucose oxidation could be reproduced by simple supplementation of suspending media with FFA raises the intriguing possibility that the mobilization of preformed substrate by TSH may, at least in adipose tissue, constitute the progenitor for the changes in respiratory and carbohydrate metabolism. Obviously, caution must be exercised in applying the findings from the fat pad to the problems of TSH action in the thyroid. For any tissue, the consequences of hormone action and substrate mobilization will be conditioned by the subcellular localization of these processes, the nature and abundance of preformed substrate, and the intrinsic biochemical potentialities of that tissue. Thus, in view of the organizational dissimilarities of thyroid and adipose tissue, it is not surprising that certain processes, such as the oxidation of assimilated glucose, are quite differently affected by TSH in these structures. However, within the framework of suggesting new avenues for thyroid investigation,

[9] Although glycogen metabolism has not been studied, analogy to findings with other hormones (35, 38) suggests that a stimulation of glycogenolysis by TSH in adipose tissue also might be anticipated.

the present studies have fulfilled experimental objectives. Whether the data justify more specific extrapolation with regard to the intrathyroidal action of TSH must be deferred for further inquiry.

SUMMARY

1. The actions of pituitary thyrotropin (TSH) upon the metabolism of rat epididymal adipose tissue have been examined. Commercial as well as the most highly purified available preparations of TSH were employed.

2. Addition of all types of TSH to the incubation mixture resulted in an increase in the tissue stores of free fatty acids, and a release of fatty acids into the suspending medium in the presence of extracellular albumin. In contradistinction to the lipolytic effects of equivalent quantities of corticotropin (ACTH), the action of TSH was not contingent upon the presence of extracellular ionic calcium.

3. Concomitant with the induction of lipolysis, TSH effected an enhancement of the oxygen consumption of adipose tissue which was maximal in the presence of extracellular glucose. Assimilation of glucose from all types of suspending media was also promoted by TSH. Heating of TSH for 3 minutes at pH 2 caused profound reduction in its effects upon lipolysis, respiration and glucose assimilation. Such mild acid-heating did not diminish the similar activities of ACTH in adipose tissue.

4. Differentially-labeled glucose-C^{14} was employed to assess the effects of TSH upon the disposition of assimilated glucose. TSH caused a disparate augmentation in the oxidation of glucose carbon-6.

5. Simple supplementation of albumin and glucose-containing media with larger quantities of palmitate or oleate effected comparable alterations in oxygen consumption and glucose oxidation. The parallelisms prompted the suggestion that lipolysis may constitute the progenitor for some of the TSH-induced changes in the metabolism of surviving adipose tissue.

6. Adipose tissue has been analyzed as a model system wherein stimulation of respiratory activity and assimilative capacity may occur coincident with mobilization of preformed endogenous substrate. The possible implications with regard to the intrathyroidal action of TSH have been discussed.

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EFFECTS OF INFUSIONS OF SERUM ALBUMIN ON SERUM LIPIDS AND LIPOPROTEINS IN NEPHROSIS

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Serum lipid and lipoprotein alterations in nephrosis were discussed in detail in a previous report (1). It was found that there were qualitative as well as quantitative differences in lipoproteins from one patient to another. Despite the differences in lipoprotein pattern, concentrations of serum cholesterol and phospholipids were related inversely to concentrations of serum albumin in a fairly regular manner. Triglycerides were consistently increased only when serum albumin fell below levels of about 1 g per 100 ml. These and other (2) observations suggest that hyperlipoproteinemia in nephrosis may be at least in part a result of hypoalbuminemia.

In order to study the effects on serum lipoproteins of altering the level of serum albumin without altering the disease, repeated infusions of serum albumin were administered to patients with nephrosis. Serum lipids and lipoproteins decreased as a result of the infusions. The decreases often were selective, involving principally the particular fractions which were most excessive at the beginning of the infusions. Changes in lipids and lipoproteins during infusions of dextran, and during steroid-induced remissions of the disease, were studied in several patients for comparison with the albumin results.

METHODS

Patients. The subjects were 9 patients with nephrosis (nephrotic syndrome) of unknown etiology. Age of the patients ranged from 5 to 46 years. Edema was present in all but one patient and proteinuria (4 to 15 g per day) was present in all cases prior to the initial course of infusions. A majority of the subjects was hospitalized, fed a low sodium diet, and studied for periods of several weeks before infusions were started. Body weight and levels of serum albumin, serum lipids, and urine protein were reasonably steady, except in Patient R.B., in whom steroid therapy had recently been discontinued, and in one additional patient who was excluded as noted later. Diet, schedule of activities, and other conditions were continued without change throughout the experimental periods. Quantity of food consumed was not rigidly controlled but did not change appreciably.

Albumin infusions. The courses of albumin with one exception consisted of a total of 175 to 1,075 g, given over periods of 7 to 26 days, in quantities of 25 or 50 g per day with a few exceptions which are noted. The albumin preparation was salt-poor Cohn fraction V in 25 per cent solution, supplied by the American National Red Cross. Each infusion was administered intravenously over a period of 1 to 2 hours, starting about 7:30 a.m., after first obtaining blood specimens for analysis if desired. Because of the rapid loss of albumin in the case of W.T., a final infusion of 90 g was given slowly over a 10 hour period.

Dextran infusions. One patient (F.G.) who had previously received albumin, was given 3 daily infusions of dextran in isotonic sodium chloride solution, and another patient (F.N.), a 30 year old male, was given 4 daily infusions of dextran in water. A third patient who received dextran, with results resembling those in F.G., was excluded because of a rapid increase in lipids just before the infusions were started. Each infusion, consisting of 30 g of dextran (average mole weight 75,000) in 6 per cent solution, was administered intravenously over a period of 6 to 8 hours.

Blood specimens and analytic procedures. Blood specimens for analysis were obtained about 7:30 a.m., after an overnight fast. Specimens during and at the end of the infusion periods were thereby taken *almost 24 hours after the end of the most recent infusion.* Shorter periods of time elapsed after the long albumin infusion in W.T. and the dextran infusions. Urine was collected continuously in 24-hour pools, starting at 7 a.m. For urine protein determinations, the infusion period was considered to extend to 7 a.m. of the day following the final infusion.

Many of the methods have been described in detail previously (1). Total lipids were determined on chloroform-methanol extracts of serum by the method of Bragdon (3). Determinations of total and free cholesterol were made by the Schoenheimer-Sperry procedure (4). Lipid phosphorus was determined by the method of Stewart and Hendry (5) and multiplied by a factor of 25 to obtain values for phospholipids. Triglycerides were calculated by difference (3), and therefore are possibly less accurate than the other lipids, particularly when triglycerides were relatively low. A modification of the dye interaction method of Rutstein,

TABLE I

Data on albumin infusions and some of their effects *

Patient and course	Age	Albumin infusions		Serum albumin		Plasma volume		Urine protein			Body weight	
				I	II	I	II/I	I	A	B	I	II
	yrs	*g*	*days*	*g/100 ml*		*L*		*g/day*	*% infused protein*		*kg*	
						Group 1						
M.C., 1	14	225	8	0.6	2.4	1.96	0.85	13	29	0	46	39†
M.C., 2	14	475	26‡	1.8	4.0	2.00	0.86	6.5	7	0	40	40†
S.J.	15	175	7	2.7	3.5	2.47	0.91	4.2	29	18	49.5	49†
						Group 2						
						A						
R.B., 1	6	100	3	1.0	1.9		0.70	10			27.5	24.5
R.B., 2	6	175	7	0.8	2.8	1.09	0.90	6	53	23	23.5	19†
W.T.		290	8	0.5	1.4		0.93	5.5	62	9	39.5	38.3
						B						
F.G.	46	650	13	1.0	2.9	2.94	0.91	15	66	15	68.5	66.2†
C.T.	30	1,075	22	0.8	3.7	2.53	0.70	13	57	8	65.5	57†
J.S., 1	5	325	14‡	0.6	1.4	0.85	0.86	8.4	56	9	25.5	24.5
J.S., 2	5	600	16‡	0.6	2.2	1.01	0.81	10	69	11	25.5	21.5†

* I = level during control period. II = level at end of infusion period (almost 24 hours after last infusion). A = cumulative excess above control level during infusion period. B = cumulative excess above control level during post-infusion period.

† Patients were approximately free of edema when these weights were recorded.

‡ Infusions were omitted on certain days (see Figures 1 and 4).

Ingenito and Reynolds (6) was employed for serum albumin analysis, and urine protein was determined by a biuret method (7). Lipoprotein fractions of serum were isolated by preparative ultracentrifugation after successive adjustments of serum density (1), and analyzed by the methods described above. Free fatty acids were determined by the method of Gordon and Cherkes (8). Determinations of serum dextran were made as described by Semple (9). Changes in plasma volume were calculated from hematocrit readings, $\Delta PV = [(Ht_1/Ht_2) - 1]/[1 - (Ht_1/100)]$, and from dye T-1824 dilution (10). Optical density read at 660 mμ in a Coleman Jr. spectrophotometer was taken as a measure of serum lactescence.

RESULTS

Effects of albumin infusions

Data on the albumin infusions and some of their effects are summarized in Table I. In Group I are courses in patients with clear serum and near normal triglycerides, while in Groups 2A and 2B are courses in patients with high levels of triglycerides as well as total cholesterol and phospholipids. Results of courses in Group 2A are possibly less significant than the others for reasons noted elsewhere.

Serum albumin concentration gradually increased in all patients but one (W.T.), and finally was increased briefly in this case by a large infusion. Proteinuria increased greatly. The maximal levels of proteinuria sometimes were approximately equal to the daily quantities of albumin infused. However, the excess protein in the urine (above the control level), during the infusion period and in the postinfusion period to the point of returning to the control level, was always less than the total quantity of albumin infused. Serum albumin concentration and proteinuria usually returned approximately to preinfusion levels within about a week after the infusions had been discontinued. An exception was the case of M.C. in whom proteinuria increased early in the infusion periods, but then decreased, and fell below control levels after the completion of each of two courses. It is evident that a partial remission of the disease occurred during the administration of albumin in this patient.

Diuresis accompanied the albumin infusions. The decrease in body weight and the increase in serum albumin usually progressed simultaneously. Complete loss of edema occurred in cases in which serum albumin levels were maintained above approximately 2 g per 100 ml. No adverse effects could be attributed to the albumin. Changes

in serum electrolytes were in the direction of normal. Serum creatinine did not change appreciably. Hypertension or evidence of cardiac failure was not observed. In some cases, there was a striking increase in energy and feeling of well-being. However, the relief of symptoms usually did not last long.

The albumin infusions undoubtedly increased plasma volume and caused at least transitory dilution of serum lipids. Dilution also followed removal of blood for analysis. Plasma dilution due to both processes presumably is reflected in the changes in hematocrit readings which were more consistent and at least as great as changes in T-1824 dilution. Ratios of initial plasma volume to final plasma volume, 24 hours after the last infusion—as indicated by changes in hematocrit or, in the case of J.S., with progressive anemia, by dye T-1824 dilution measurements—are shown in Table I. Reductions in concentration of lipoproteins as a consequence of chronic dilution alone probably would not have persisted very long because of the relatively short turnover time of lipoproteins. This is emphasized by the failure of repeated plasmapheresis to decrease plasma lipids in animals (11). It is likely, therefore, that the effects of plasma volume changes per se on lipid concentrations, particularly in the courses of longer duration, were not great. The data presented are observed values, not adjusted for changes in plasma volume, with a few exceptions which are noted.

Effects of albumin infusions on serum lipids

Some or all of the serum lipids decreased in all cases during the albumin infusions, as may be seen in Table II. The changes during six of the courses are shown in detail in Figures 1–4.

Total cholesterol (TC) decreased by 31 to 60 per cent during all but one course, with no consistent change in ratio of free to total cholesterol. Phospholipids (PL) also decreased in all cases, but to a lesser degree. Ratios of TC/PL, which initially were high, changed toward normal during the infusions, and then increased again after the infusions had been discontinued. Note the convergence and crossing of TC and PL levels during courses shown in Figures 1–4. TC and PL

TABLE II

Changes in serum lipids during albumin infusions *

Patient and course	TL		TC			PL			TG			TC/PL	
	I	II/I	I	II	II/I	I	II	II/I	I	II	II/I	I	II
	mg/100 ml		*mg/100 ml*			*mg/100 ml*			*mg/100 ml*				
Approx. normal values†	580		180			225			100			0.80	
Group 1													
M.C., 1	1,703	0.78	676	446	0.66	538	409	0.76	158	256	1.62	1.26	1.09
M.C., 2	986	0.66	373	204	0.55	308	210	0.68	115	137	1.19	1.21	0.97
S.J.	999	0.76	373	258	0.69	325	246	0.76	107	124	1.16	1.15	1.05
Group 2													
A													
R.B., 1	2,985	0.77‡	837	570‡	0.68‡	835	629‡	0.75‡	900	814‡	0.90‡	1.00	0.91
R.B., 2	2,132	0.87	702	571	0.81	578	488	0.84	550	520	0.95	1.21	1.17
W.T.	3,398	0.86	825	517	0.63	568	470	0.83	1,615	1,695	1.05	1.45	1.10
B													
F.G.	2,652	0.62	659	414	0.63	633	436	0.69	1,058	618	0.58	1.04	0.95
C.T.	2,231	0.48	639	257	0.40	456	266	0.58	825	419	0.51	1.40	0.97
J.S., 1	2,593	0.53	736	357	0.49	553	353	0.64	950	506	0.53	1.33	1.01
J.S., 2	2,606	0.44	611	346	0.57	532	335	0.63	1,179	305	0.26	1.15	1.03

* TL = total lipids; TC = total cholesterol; PL = phospholipids; TG = triglycerides. I = level before infusions were started; II = level at end of infusion period (almost 24 hours after last infusion).

† Approximate normal values for young adults, discussed previously (1).

‡ Because course R.B., 1 was only 3 days long, the lipid values recorded here for Period II are the observed values corrected for the change in plasma volume (× 1.43). All other values in the table are observed values.

reached near normal concentrations in the three instances in which serum albumin rose to levels of at least 3.5 g per 100 ml. On the other hand, the quantitative relationship between serum albumin and serum lipids was not consistent.

The relationship between triglycerides (TG) and other lipids in the period before infusions were started differed considerably in different patients, as noted in an earlier study (1). In two patients with normal TG (M.C. and S.J.) and in two with high TG (R.B. and W.T.), there was little or no decrease in TG during the albumin infusions. The failure of TG to fall in the latter two cases (Group 2A) does not seem to be in accord with results in other cases, and may have been due in part to the small quantities of albumin administered, together with effects of recent steroid therapy in R.B., and the very rapid loss of albumin by W.T. In the remaining cases, all with high levels of TG initially, albumin infusions caused large decreases (42 to 74 per cent) in TG. Serum lactescence in these cases decreased at least in proportion to the fall in TG. It is evident that the individual lipids were affected differently in different patients by albumin, depending in part upon the pattern of lipids (lipoproteins) present at the beginning of the infusions.

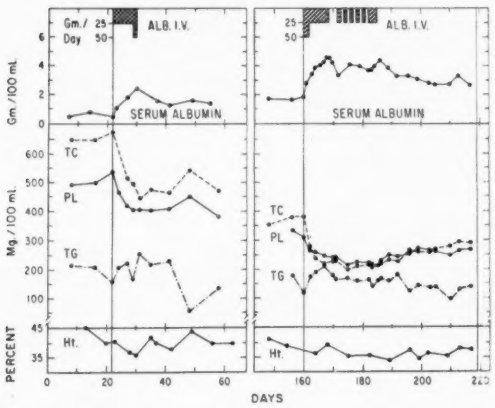

FIG. 1. EFFECTS OF ALBUMIN INFUSIONS (TWO COURSES) ON SERUM LIPIDS IN PATIENT M.C.

Free fatty acids. Free fatty acids in the serum (FFA) were determined frequently at 7:30 a.m. throughout courses of albumin infusions in three patients (M.C., C.T. and W.T.). Levels of FFA

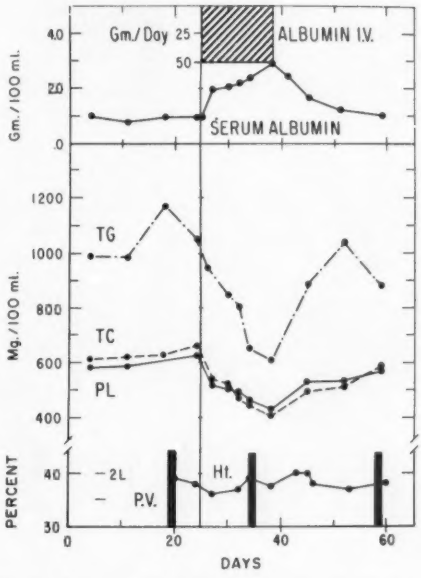

FIG. 2. EFFECTS OF ALBUMIN INFUSIONS ON SERUM LIPIDS IN PATIENT F.G.

in these (and other) cases during control periods were normal (0.25 to 0.7 mEq per L), in agreement with observations of Shafrir (12). Molar ratios of FFA/albumin were usually greater than

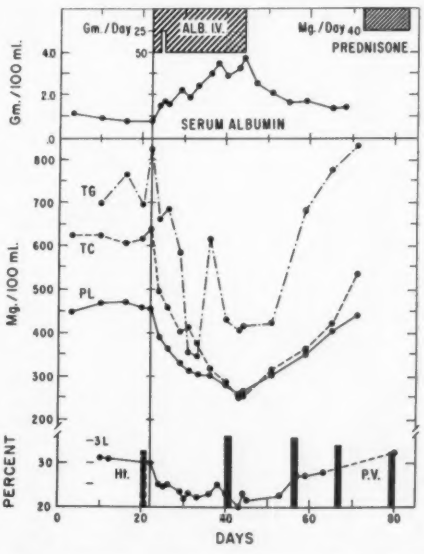

FIG. 3. EFFECTS OF ALBUMIN INFUSIONS ON SERUM LIPIDS IN PATIENT C.T.

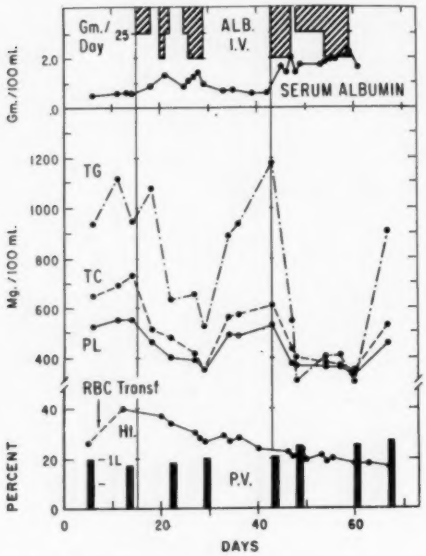

FIG. 4. EFFECTS OF ALBUMIN INFUSIONS ON SERUM LIPIDS IN PATIENT J.S.

normal. FFA increased somewhat during the infusions in one case (M.C.) and probably also in another (W.T.), although no concentrations as great as 1.0 mEq per L were observed. In the patient in whom there was the greatest decrease in lipids (C.T.), no definite change in FFA occurred. In two patients (C.T. and W.T.) FFA did not increase during remissions of the disease induced by prednisone.

Effects of albumin infusions on lipoproteins

Serum lipoproteins separated by centrifugation (1) were studied during the courses of albumin. In M.C. and S.J., both with normal TG initially, the density (D) 1.019–1.063 lipoprotein fraction decreased to a somewhat greater degree than did the very low density (D < 1.019) fraction. This probably also occurred in R.B. and W.T., although the lipoprotein data were inadequate in these cases. In the remaining patients, all with high levels of D < 1.019 lipoproteins, the predominant change was a decrease in the D < 1.019 fraction. It should be noted that decreases in this fraction involved very significant decreases not only in TG but also in TC, PL and TC/PL. The results are shown in Table III and in Figures 5 and 6. Values for the high density (D 1.063–1.21) lipoproteins probably are much less accurate than the others because of their relatively small size and their liability to error as a result of inaccuracies in separating the other fractions. The lipoprotein changes were in accord with serum lipid changes described above. The addition of albumin to lactescent sera *in vitro* did not affect lactescence or the distribution of lipids or protein among the lipoprotein fractions of different densities.

Effects of dextran infusions on serum lipids and lipoproteins

Results of dextran infusions in two patients are summarized in Figures 7 and 8. Because the

TABLE III

Changes in lipids of serum lipoprotein fractions during albumin infusions in three patients with lactescent serum (Group 2B) *

Patient and course	D <1.019						D 1.019–1.063				D 1.063–1.21			
	TC		PL		TG		TC		PL		TC		PL	
	I	II/I	I	II/I	I	II/I	I	II/I	I	II/I	I	II/I	I	II/I
	mg per 100 ml of serum													
Approx. normal values*	25		30		80		100		75		50		100	
F.G.	404	0.48	379	0.60	903	0.57	212	0.80	190	0.79	12	1.00	30	1.10
C.T.	507	0.30	341	0.41	799	0.38	103	0.87	83	0.77	24	0.71 (0.96)†	28	0.58 (0.88)
J.S., 1	412	0.29	336	0.42	1,013	0.35	216	0.92	164	0.92	9	1.12	22	1.36

* See footnotes to Table II.

† The results in parentheses were obtained 1 week after albumin had been discontinued, at a time when the lipids of other fractions were only slightly higher than the levels of Period II.

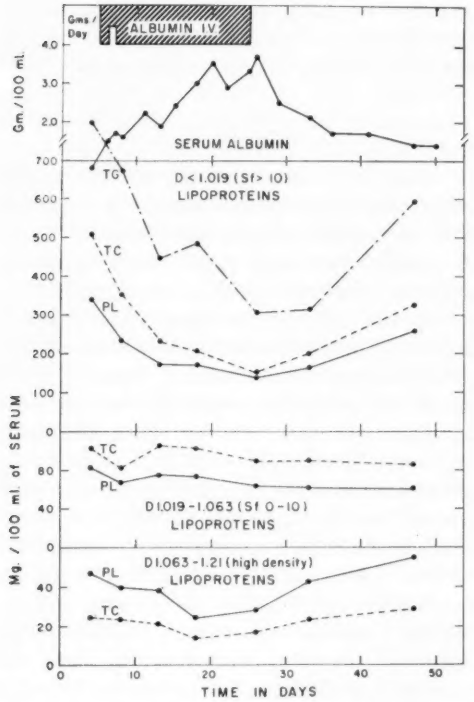

FIG. 5. EFFECTS OF ALBUMIN INFUSIONS ON LIPIDS IN SERUM LIPOPROTEIN FRACTIONS OF PATIENT C.T.

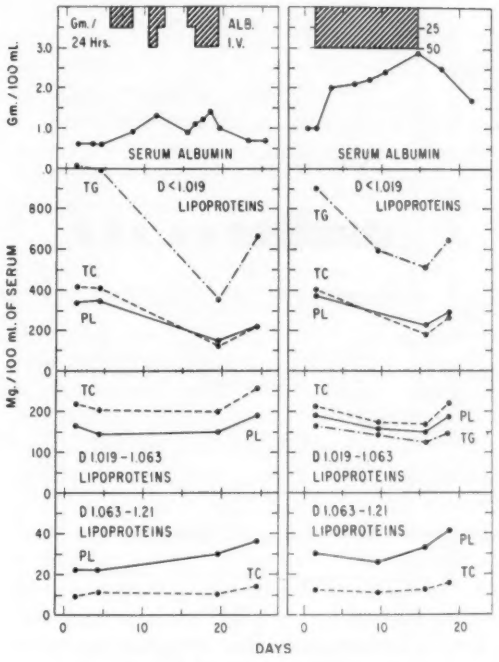

FIG. 6. EFFECTS OF ALBUMIN INFUSIONS ON LIPIDS IN SERUM LIPOPROTEIN FRACTIONS OF PATIENT J.S. (LEFT FRAME) AND PATIENT E.G. (RIGHT FRAME).

courses were brief, concentrations of serum lipids and albumin shown in Figures 7 and 8 after the beginning of infusions have been increased above observed values in proportion to plasma dilution as indicated by changes in hematocrit at each point. Substantial decreases in lipids occurred. In the case of F.N., with near normal TG and clear serum initially, there was little change in TG but definite decreases in TC and PL. In F.G., with high TG and lactescent serum, large decreases in TG as well as in TC and PL resulted. Ratios of TC/PL fell slightly in both instances. The lipid changes persisted after dextran had been largely eliminated from the serum. The lipoprotein changes were in accord with the serum lipid changes and resembled those induced by albumin. In F.N. there was a decrease in the D 1.019–1.063 fraction, while the principal change in F.G. was a fall in the D < 1.019 fraction. Changes in quantity of the lipoprotein fractions as indicated by PL content are shown in Table IV.

Absence of any appreciable increase in FFA throughout the day of an infusion in the third patient who received dextran (course not shown), and on successive days of infusions in F.N. (Figure 7), suggests that dextran had little if any lipoprotein lipase activity. Dextran caused no complications except for oliguria following the infusions in the third patient. Proteinuria increased significantly during two courses of dextran, in accord with previous observations (13).

TABLE IV

Changes in phospholipid of serum lipoprotein fractions during infusions of dextran *

	D < 1.019			D 1.019–1.063			D 1.063–1.21		
	PL			PL			PL		
	I	II	II/I	I	II	II/I	I	II	II/I
	mg per 100 ml of serum								
F.N.	77	80	1.04	202	131	0.65	49	50	1.02
F.G.	321	210	0.65	153	154	1.01	23	16	0.70

* I = level before dextran infusions. II = level 9 days after dextran infusions.

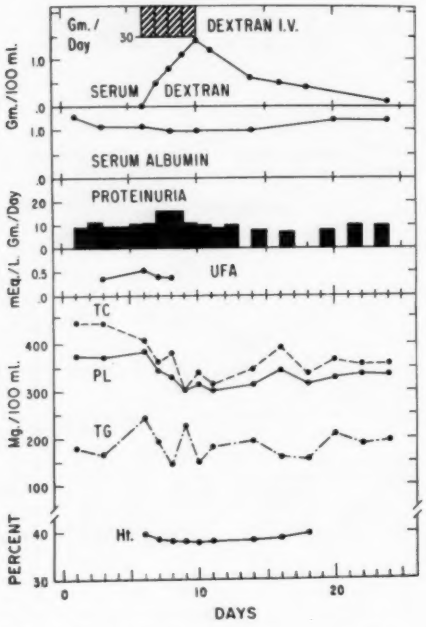

FIG. 7. EFFECTS OF DEXTRAN INFUSIONS ON SERUM LIPIDS IN PATIENT F.N.

Serum lipids and lipoproteins during changes in the disease

In spontaneous remissions and exacerbations of nephrosis, and in remissions induced by prednisone therapy, serum cholesterol usually changed inversely with serum albumin. Albumin sometimes seemed to change first, but usually the alterations in albumin and cholesterol were very nearly simultaneous. Prednisone usually caused an increase in cholesterol prior to and in the absence of remissions. Changes in PL and TG during steroid therapy have not been studied except as noted below.

Very early in remissions of the disease induced by prednisone in several patients, lipoprotein alterations consisting of a fall in D < 1.019 lipoproteins (with a decrease in serum TG and lactescence) together with a rise in the D 1.019–1.063 fraction occurred prior to any great increase in serum albumin concentration. As serum albumin increased, the D 1.019–1.063 fraction fell. The initial lipoprotein changes in these cases possibly were caused by metabolic effects of the steroid and not by changes in the disease, but the observations emphasize the fact that lipoproteins in nephrosis may be greatly influenced by factors other than serum albumin concentration.

DISCUSSION

Previous observations already referred to suggested that hyperlipoproteinemia in nephrosis might be caused by hypoalbuminemia. On the other hand, serum lipids usually are not increased in portal cirrhosis (14), malnutrition (15), kwashiorkor (16), or the hypercatabolic type of idiopathic hypoalbuminemia (17) in which low levels of serum albumin occur. Possibly this is because of associated nutritional or metabolic disturbances in these diseases when albumin is very low. In the ananabolic type of idiopathic hypoalbuminemia, serum cholesterol is consistently increased and decreases when albumin is infused (17), but the degree and type of lipid alterations and also of edema resemble those seen in nephrosis with higher levels of serum albumin.

In the present study, albumin infusions in patients with nephrosis caused serum lipids and lipoproteins to decrease. Decreases in individual

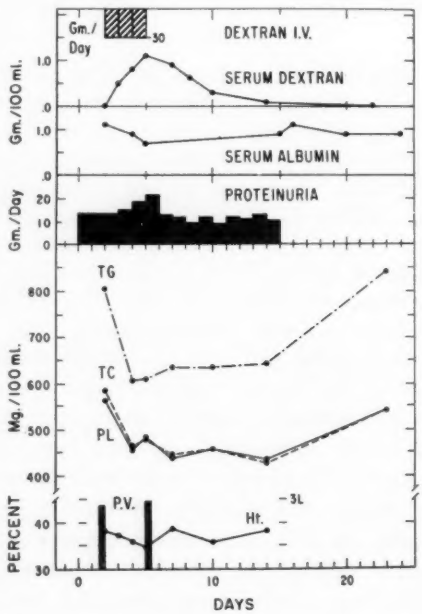

FIG. 8. EFFECTS OF DEXTRAN INFUSIONS ON SERUM LIPIDS IN PATIENT F.G.

cases did not involve all of the fractions equally. The results could not be explained by simple plasma dilution. However, lipid abnormalities seldom disappeared entirely, and certain ones at times changed little. Further and more consistent corrections might have occurred if satisfactory replacement of albumin had been accomplished more consistently. The magnitude of the effects is difficult to evaluate, but it appeared that lipoprotein metabolism was altered by the albumin infusions. The pathogenesis of nephrotic hyperlipoproteinemia is not necessarily elucidated by the observations, since the reduction in lipids might have resulted from effects of the infusions other than replacement of deficient albumin.

Since free fatty acids are normally bound principally to albumin in the serum (12), the lipid abnormality in nephrosis might conceivably be related to a disturbance in transport or metabolism of FFA due to low serum albumin. Such a mechanism was considered by Gitlin and associates (18) as a possible explanation of their observation that conversion of S_f 10–200 lipoproteins to those of S_f 3–9 was decreased in nephrotic children. Depression of FFA by 48-hour infusions of glucose, however, failed to cause any great reduction in serum lipids in nephrotic subjects (19).

Peters and Man (20) noted large decreases in serum lipids following albumin infusions in hypoalbuminemic patients with and without nephrosis, whether lipids were originally high or low. There were indications that the changes were not caused by hemodilution. A 50 per cent fall in cholesterol during albumin infusions was observed more recently by Soothill and Kark (21), and was attributed to a decrease in hepatic synthesis of cholesterol occurring in connection with a presumed decrease in albumin synthesis. Allen (22) noted that lipids in nephrotic rats were changed toward normal by injections of various macromolecular substances in addition to albumin. Dextran had previously been observed to decrease serum cholesterol in nephrotic patients (23) and animals (24) and also in normal animals (25). Albumin synthesis appears to be decreased by dextran (26, 27). There is no assurance that albumin and dextran act in the same manner. However, the observation that dissimilar substances of large molecular size share the lipid-reducing effect, suggests that the reduction may be mediated through changes in colloidal osmotic pressure or volume of the plasma.

The effects of albumin infusions on serum albumin concentration, proteinuria and edema were similar to those generally noted by previous investigators (28–32).

SUMMARY

Ten courses of serum albumin, each usually consisting of 25 to 50 g daily for 1 to 4 weeks, were administered intravenously to seven patients with nephrosis. Serum albumin concentration and proteinuria increased. Complete loss of edema occurred in a majority of instances.

Total serum cholesterol (TC), phospholipids (PL), and ratio of TC/PL, all of which were initially high, fell consistently during the albumin infusions. Serum triglycerides (TG) and lactescence decreased greatly in 3 of 5 patients with initially high levels of TG. In the same three cases there was a selective fall in lipoproteins of density < 1.019. No decrease in TG occurred in two patients with near normal levels of TG prior to the infusions. Free fatty acids changed inconsistently.

Dextran appeared to induce lipoprotein changes which were similar to those obtained with albumin. Prednisone caused lipoproteins to change prior to any considerable change in serum albumin. The extent and means by which lipids and lipoproteins in nephrosis are altered by albumin, and implications regarding the cause of the lipid abnormality in nephrosis, are discussed.

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EFFECTS OF DEXTRAN, POLYVINYLPYRROLIDONE AND GAMMA GLOBULIN ON THE HYPERLIPIDEMIA OF EXPERIMENTAL NEPHROSIS *

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During the treatment of edema in nephrotic patients by the infusion of dextran, Mollison and Rennie (1) noted falls in serum cholesterol concentration which they believed were due to dilution but which persisted after hematocrits had returned to pretreatment levels, and which by calculation from their data were greater than could be accounted for by hemodilution alone. Subsequently Soothill and Kark (2) and Baxter, Goodman and Havel (3) demonstrated that the intravenous infusion of human albumin into nephrotic patients resulted in a significant lowering of serum lipid concentrations. In their studies on nephrotic rats Rosenman and Friedman (4) demonstrated that the infusion of bovine albumin into these animals lowered serum lipid concentrations. Heymann, Nash, Gilkey and Lewis (5) treated nephrotic rats with dextran and noted significant reductions in the serum concentrations of total lipid and cholesterol. They recognized that these reductions resulted from more than hemodilution, but offered no explanation for this effect. This study was undertaken to investigate more extensively the effects of the administration of dextran on the hyperlipidemia of experimental nephrosis in rats, and to see whether these effects were shared by osmotically active macromolecular substances other than dextran and albumin.

METHODS

Female rats of the Osborne-Mendel strain, weighing approximately 200 g, were made nephrotic by the intravenous injection of an aliquot of pooled antirat-kidney rabbit serum prepared as previously reported from this laboratory (6). Eleven days after injection of the antiserum a control blood sample of approximately 1.2 ml volume was obtained by heart puncture after a 6 hour fast. On Days 12, 13 and 14 after injection of the antiserum the rats received, twice daily, intraperitoneal injections of dextran, polyvinylpyrrolidone (PVP), bovine γ-globulin, bovine albumin or salt in solutions as described below. On Day 15 following antiserum injection, approximately 20 hours after the last intraperitoneal injection and again after a 6 hour fast, the final blood sample was obtained by aspiration from the exposed abdominal aorta. Both heart puncture and aortic aspiration were done under ether anesthesia. Both blood samples were obtained through clean, dry needles into heparinized syringes. Normal control animals were handled as were the nephrotic rats except for the initial injection of antiserum. The animals were housed in metabolic cages with free access to water and Purina pellet diet, save for the presampling fasting periods when pellets were withheld. Twenty-four-hour urine collections were made from Days 11 through 15 after the injection of the antiserum and were used for the determination of urinary protein content.

Hematocrits were determined in duplicate on each blood sample by the capillary microhematocrit method immediately after each sample was obtained. Because of the observed ability of dextran and PVP to increase the erythrocyte sedimentation rate, careful mixing and rapid loading of the capillary tubes were insured. These hematocrits were used for calculation of plasma volume changes during the experimental period by the formula

$$\text{fractional change in plasma volume} = \frac{\text{plasma volume}_2 - \text{plasma volume}_1}{\text{plasma volume}_1} = \frac{(\text{hematocrit}_1/\text{hematocrit}_2) - 1}{1 - (\text{hematocrit}_1/100)}$$

where hematocrit_1 and hematocrit_2 represent the average hematocrits obtained at the first and second blood samplings, respectively. Plasma concentrations of albumin and lipids obtained following treatment with the macromolecular substances have been corrected for such plasma volume changes. The calculation presented in the formula above is based on the assumption that the circulating red cell mass remains constant during the experimental period. A decrease in the red cell mass during treatment would lead to overcorrection, thus minimizing the changes shown by these data. A few animals had evidence of intraperitoneal, gastrointestinal

* A preliminary report of this work appeared as an abstract (Clin. Res. 1959, 7, 278).

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or external bleeding as a result of the injections; these were not included in the study.

Plasma was separated from the blood samples by centrifugation and stored at 5° C in stoppered containers for 1 to 4 days before lipid extraction and the various other determinations described below. Before aliquots were taken for analysis, the plasma samples were brought to 37° C and mixed carefully by inversion. Aliquots were extracted with chloroform-methanol by the method of Havel (7). Aliquots of the extracted lipids were used for the determination of total lipids by the method of Bragdon (8), total cholesterol by the method of Zak and co-workers (9), and phospholipids by a modification of the method of Stewart and Hendry (10), using a factor of 25 for the conversion of lipid phosphorus to phospholipid. Only those nephrotic rats with initial total lipid concentrations of 500 mg per 100 ml or greater (approximately twice the mean normal value in this laboratory) were used in the study. All of the methods were standardized for microdeterminations. Plasma albumin concentrations were determined by a modification of the dye interaction method of Rutstein, Ingenito and Reynolds (11). Determinations of dextran (12) and PVP (13) were performed on the appropriate samples. Total plasma protein concentrations and urinary protein content were determined by modifications of the method of Hiller, Greif and Beckman (14).

By the method of Bragdon (8) plasma triglyceride concentration may be determined by appropriate calculations from plasma total lipid, phospholipid, and total and free cholesterol concentrations. Because of the small volume of plasma available, free cholesterol determinations were not performed on the experimental animals. In separate groups of normal and nephrotic rats, however, free and total cholesterol concentrations were determined by a modification of the Schoenheimer-Sperry method (15). Free cholesterol to total cholesterol ratios of the nephrotic and normal groups were, respectively, 0.28 (SD 0.01) and 0.32 (SD 0.01). By use of these ratios the plasma concentration of free cholesterol was approximated in each animal from the total cholesterol value, and triglyceride concentrations were then calculated. It is recognized that the free cholesterol to total cholesterol ratio may have changed following treatment with these macromolecular substances, but a significant change in this ratio will introduce only a small error into the calculated triglyceride concentration.

Both extraction procedures and chemical determinations were checked for reliability in the presence of the various macromolecular substances used in this study by *in vitro* addition and duplicate determination. No interference was demonstrated except in the determination of total lipids by the dichromate-reduction method of Bragdon in the presence of PVP. Various extraction methods were tried in an effort to remove PVP from the lipid phase, all of which were unsuccessful owing to the solubility of that compound in both polar and nonpolar solvents. Hence, for the animals treated with PVP, total lipid concentrations were derived from determinations of plasma carboxylic acid ester content done by a modification of the method of Rapport and Alonzo (16). In a separate group of nephrotic rats, total lipid determinations by the two methods showed the dichromate reduction method to give values approximately 1.5 times those determined by the ester method. This ratio cannot, of course, be justified for values obtained after PVP treatment. Triglyceride concentrations for PVP-treated rats are not presented, since they cannot be compared with those calculated from dichromate-reduction total lipid determination.

Dextran was administered as a commercially supplied 6 per cent solution in isotonic saline (Gentran, Baxter Laboratories, Inc.) in amounts of 0.3 to 1.4 g per day. The dextran in this preparation has an average molecular weight of 75,000. Commercially prepared powdered PVP (fraction K-30 from General Aniline and Film Corp.) was fractionated by the isopropanol-acetone method outlined by Levy, Caldas and Fergus (17), to obtain a sample with an average molecular weight of 75,000 as determined by viscosity measurement. This was dissolved to a 6 per cent solution in isotonic saline and injected intraperitoneally in amounts of 0.2 to 1.0 g per day. Bovine albumin prepared by Armour Pharmaceutical as bovine plasma fraction V was administered as 10 to 20 per cent solutions in isotonic saline in amounts of 1.2 to 4.0 g per day. Bovine γ-globulin (Armour) prepared as bovine plasma fraction II was administered as 15 to 20 per cent solutions in isotonic saline in amounts of 2.0 to 4.0 g per day. A group of nephrotic rats received twice daily intraperitoneal injections of isotonic saline solution in a volume equal to the largest volume of injection received by any of the experimental animals (9 ml). In general, normal control rats received smaller amounts of the various agents than their nephrotic counterparts, but achieved comparable plasma concentrations owing to the smaller urinary loss.

Lipoprotein lipase activity was assayed in both normal and nephrotic rats following the intravenous injection of 1 mg of heparin, 3 to 150 mg of dextran, 90 mg of PVP, and 195 mg of γ-globulin. All injections were made in a standard volume of 1 ml and the amounts administered were calculated to provide plasma concentrations comparable with those determined following the intraperitoneal treatments outlined above. Fifteen minutes after the intravenous injections the rats were bled into heparinized syringes from the abdominal aorta under ether anesthesia. The plasma was separated immediately by centrifugation, and 1-ml aliquots were added to cuvets containing a commercial coconut oil emulsion (Ediol, Schenley Laboratories) diluted 1 to 50 with normal saline. The cuvets were held in a water bath at 37° C and optical densities were read in a Coleman Jr. spectrophotometer at 660 mμ immediately and at 5-minute intervals for up to 140 minutes. Clearing was followed in duplicate experiments with and without the *in vitro* addition of 25 to 30 mg of albumin in the form of a 15 per cent solution of Armour fraction V bovine albumin. Clearing activity was also assayed in post-heparin plasma following the *in vitro* addition of 30 mg of dextran or PVP. Plasma obtained from rats follow-

TABLE I

Mean plasma concentrations attained by the intraperitoneal administration of dextran, polyvinylpyrrolidone (PVP), γ-globulin and albumin to nephrotic and normal rats

Substance injected	Substance determined	Concentrations			
		Nephrotic rats*		Normal rats*	
		Before	After	Before	After
		g/100 ml plasma			
Dextran	Dextran	0	1.6 ± 0.2 † (0.3 − 3.0)‡	0	1.6 ± 0.1 (1.3 − 2.1)
PVP	PVP	0	1.7 ± 0.1 (1.2 − 2.5)	0	1.4 ± 0.1 (1.1 − 1.7)
γ-Globulin	Total protein	4.9 ± 0.2 (3.7 − 6.2)	7.3 ± 0.2 (6.2 − 8.9)	6.8 ± 0.2 (6.5 − 7.0)	9.7 ± 0.3 (8.5 − 10.2)
Albumin	Albumin	1.8 ± 0.2 (1.1 − 3.4)	2.5 ± 0.2 (1.4 − 3.9)		

* All p values for significance of the difference between before and after concentrations are <0.01.
† Standard error of the mean.
‡ Range of values.

ing 3 days of twice daily intraperitoneal injections of dextran or PVP was also checked for clearing activity. All readings were corrected to a control optical density of 0.276.

Statistical analysis of the data to be presented was performed by the methods outlined by Stearman (18).

RESULTS

Animals. In general, both nephrotic and normal rats tolerated the injections and the macromolecular substances well. The reported idiosyncrasy of rats to dextran (19) was not observed during these experiments. Most of the animals showed no gross change in food intake and appeared active and healthy throughout the experiment. A number (representing less than one-fourth of the experimental animals) which received the largest volumes by injection appeared lethargic and had roughened fur and diminished food intake. Body weights increased slightly over the 3-day injection period. At the time of the final bleeding the abdominal cavities were inspected. In no animal was there evidence of intraperitoneal infection, and the peritoneal surfaces remained smooth and glistening. As mentioned, a few animals had evidence of intraperitoneal, retroperitoneal or gastrointestinal bleeding consequent to trauma at the time of an injection; such animals were not considered in the following data. Except for the small percentage of animals that received the largest volumes by intraperitoneal injection, absorption of the administered solutions was grossly complete at the time of the final bleeding. Average 24-hour urine volumes of the various groups increased 1.1 to 1.5 times the control volume.

Effects of the administered substances. As can be seen from Table I the intraperitoneal administration of dextran and PVP to nephrotic rats resulted in mean plasma concentrations of these macromolecules of between 1 and 2 g per cent. It is true, of course, that rapid clearance of the smaller molecules by the nephrotic kidneys resulted in a circulating macromolecular population with a higher average molecular weight than that administered. In those animals receiving γ-globulin there were significant increases in total plasma

TABLE II

Mean hematocrits before and after treatment of nephrotic and normal rats with dextran, PVP, γ-globulin, albumin or saline

Treatment	Hematocrits		
	Before	After	p*
	% by volume		
Nephrotic rats			
Dextran	39.7 ± 0.7†	34.5 ± 1.2	<0.01
PVP	40.3 ± 0.8	30.8 ± 1.0	<0.01
γ-Globulin	40.7 ± 1.0	31.2 ± 0.7	<0.01
Albumin	40.3 ± 1.0	38.6 ± 2.0	>0.05
Saline	40.1 ± 2.0	39.8 ± 2.0	>0.05
Normal rats			
Dextran	50.3 ± 1.0	39.0 ± 0.5	<0.01
PVP	51.6 ± 2.4	36.9 ± 1.1	<0.01
γ-Globulin	52.7 ± 1.5	32.8 ± 0.8	<0.01

* Significance of the difference between before and after values.
† Standard error of the mean.

TABLE III

Mean plasma lipid concentrations in normal and nephrotic rats before and after treatment with dextran, PVP, γ-globulin, albumin and saline

Treatment	No. of animals	Triglycerides			Total cholesterol			Phospholipids			T.C./P.L.*		
		Before	After†	p‡	Before	After	p	Before	After	p	Before	After	p
		mg/100 ml plasma			*mg/100 ml plasma*			*mg/100 ml plasma*			*mg/100 ml plasma*		
Nephrotic rats													
Dextran	28	572 ± 64§	133 ± 28	<0.01	332 ± 21	164 ± 20	<0.01	421 ± 22	198 ± 20	<0.01	0.78 ± 0.02	0.80 ± 0.02	>0.05
PVP	14				407 ± 29	87 ± 15	<0.01	513 ± 36	130 ± 18	<0.01	0.79 ± 0.01	0.66 ± 0.02	<0.01
γ-Globulin	12	879 ± 137	402 ± 40	<0.01	434 ± 27	284 ± 18	<0.01	542 ± 31	395 ± 26	<0.01	0.80 ± 0.02	0.72 ± 0.02	0.05 > p >0.01
Albumin	12	730 ± 165	357 ± 41	0.05 > p >0.01	341 ± 32	224 ± 14	<0.01	428 ± 38	290 ± 12	<0.01	0.79 ± 0.02	0.77 ± 0.02	>0.05
Saline	9	403 ± 79	544 ± 127	>0.05	303 ± 58	333 ± 62	>0.05	364 ± 45	396 ± 46	>0.05	0.79 ± 0.05	0.80 + 0.05	>0.05
Normal rats													
Dextran	10	46 ± 5	32 ± 5	>0.05	83 ± 5	92 ± 6	>0.05	108 ± 4	112 ± 9	>0.05	0.76 ± 0.02	0.83 ± 0.02	0.05>p >0.01
PVP	6				79 ± 3	51 ± 16	<0.01	124 ± 10	89 ± 10	0.05 > p >0.01	0.65 ± 0.03	0.56 ± 0.03	>0.05
γ-Globulin	5	35 ± 6	78 ± 10	0.05 > p >0.01	85 ± 7	135 ± 8	<0.01	123 ± 6	184 ± 11	<0.01	0.69 ± 0.02	0.73 ± 0.01	>0.05

* Mean ratios of the plasma concentrations of total cholesterol to phospholipid.
† "After" values corrected for plasma volume change before averaging.
‡ Significance of the difference between "before" and "after" values.
§ Standard error of the mean.

protein concentration, the increment presumably contributed by the injected globulin. The small mean increase in plasma albumin concentration noted here is undoubtedly a function of the rapid clearance of circulating albumin in these nephrotic animals. Plasma concentrations of these substances following their injection into normal rats were of comparable mean value, although narrower in range.

That the plasma concentrations attained were effective in increasing plasma volume, as indicated by a fall in hematocrit over the treatment period, is shown in Table II. There were statistically significant falls in hematocrit in all groups except the nephrotic rats which received albumin or isotonic saline. No significant change in hematocrit was observed unless the plasma albumin concentration increased by 1 g per cent or more, or the plasma dextran concentration was 1 g per cent or greater. Injection of γ-globulin and PVP caused significant plasma volume expansion in all of the animals presented in this data. Although it was not determined in this study, plasma concentrations of the various macromolecular substances were undoubtedly higher in the hours immediately after each injection than those represented in Table I. One would expect that even in those animals with the lowest final plasma concentrations of dextran and albumin there was some degree of plasma volume expansion following each injection.

The data in Table III demonstrate that the administration of dextran, PVP, γ-globulin and albumin to nephrotic rats resulted in significant decreases in the plasma concentrations of triglyceride, total cholesterol, and phospholipids. The injection of isotonic saline solution intraperitoneally into nephrotic rats caused no significant changes in the plasma concentrations of these lipid fractions. When administered to normal rats, dextran caused no significant changes in the concentration of triglyceride, total cholesterol or phospholipids, while PVP significantly lowered total cholesterol and phospholipid concentrations. The administration of γ-globulin to normal rats apparently caused an increase in the concentrations of these lipid fractions. Although this increase may have been due to increased lipidemia resulting from the γ-globulin treatment, it probably represents overcorrection of the final plasma lipid concentrations for plasma volume change. The intraperitoneal administration of 6 mg of dextran per day to three nephrotic animals resulted in no change in their hematocrit or plasma lipid concentrations, and no circulating dextran could be demonstrated at the time of the final bleeding.

TABLE IV

Mean fractional decreases in plasma lipid concentrations of nephrotic and normal rats treated with dextran, PVP, γ-globulin or albumin

Treatment	Fractional decrease*		
	Triglycerides	Total cholesterol	Phospholipids
Nephrotic rats			
Dextran	0.76 ± 0.04†	0.52 ± 0.04	0.53 ± 0.04
PVP		0.79 ± 0.03	0.74 ± 0.03
γ-Globulin	0.49 ± 0.08	0.33 ± 0.04	0.25 ± 0.05
Albumin	0.31 ± 0.12	0.30 ± 0.07	0.28 ± 0.06
Normal Rats			
PVP		0.36 ± 0.09	0.27 ± 0.09

* Calculated as the ratio of the change in concentration over the experimental period to the control concentration. Post-treatment concentrations were corrected for plasma volume change.

† Standard error of the mean.

Table IV summarizes the fractional changes in the various lipid moieties calculated as the ratio of their change in concentration to their original concentration. It can be seen that following injection of dextran and γ-globulin most marked changes had occured in the plasma concentration of triglyceride. This selective effect on plasma triglyceride was also demonstrated following administration of albumin to those nephrotic rats with the highest triglyceride concentration. This finding is not apparent from the mean values in Table IV owing to the wide variation in control plasma triglyceride concentration in the albumin-treated group, and the variable response of those animals with only slightly elevated triglyceride levels. Table IV also demonstrates that decreases in plasma total cholesterol and phospholipid concentration during administration of PVP were proportionally greater in nephrotic than in normal animals. When carboxylic acid ester total lipid concentration was converted to dichromate total lipid concentration (see Methods), the subsequent calculation of triglyceride concentration in a number of animals indicated that PVP, as well

TABLE V

Mean proteinuria and plasma albumin concentrations in nephrotic and normal rats treated with dextran, PVP, γ-globulin, albumin or saline

Treatment	Proteinuria			Plasma albumin conc.		
	Control	During	p*	Before	After†	p*
	mg protein/hr			*g/100 ml plasma*		
Nephrotic rats						
Dextran	14.0 ± 1.2	12.2 ± 1.2	0.05 > 0 >0.01	2.1 ± 0.1	2.3 ± 0.1	>0.05
PVP	16.4 ± 1.5	14.6 ± 1.2	>0.05	1.7 ± 0.1	2.0 ± 0.1	<0.01
γ-Globulin	18.4 ± 2.3	35.0 ± 3.5	<0.01	1.6 ± 0.2	1.9 ± 0.1	0.05 > p >0.01
Albumin	16.9 ± 2.1	51.1 ± 4.1	<0.01	1.8 ± 0.2	2.5 ± 0.2	<0.01
Saline	13.6 ± 1.8	16.6 ± 1.6	0.05 > p <0.01	1.8 ± 0.2	1.9 ± 0.2	>0.05
Normal rats						
Dextran	0.1 ± 0.0	0.2 ± 0.0	>0.05	4.9 ± 0.1	5.5 ± 0.2	0.05 > p >0.01
PVP	0.2 ± 0.0	0.2 ± 0.0	>0.05	4.9 ± 0.4	5.5 ± 0.8	>0.05
γ-Globulin	0.2 ± 0.0	0.2 ± 0.0	>0.05	5.4 ± 0.1	6.2 ± 0.3	>0.05

* Significance of the difference between before and after or during values.
† "After" values corrected for plasma volume change before averaging.

as the other macromolecular substances, lowered plasma triglyceride more than plasma total cholesterol or phospholipid. This calculation in many of the animals, however, yielded impossible (i.e., negative) values, indicating the invalidity in this method of applying the same ratio for conversion of lipid concentrations both before and after PVP treatment. The ratios of total cholesterol to phospholipid concentration (Table III) showed in general only moderate and varying changes following administration of these substances. After treatment of nephrotic rats with PVP, however, there was a significant fall in this ratio.

TABLE VI

*Mean fractional decreases * in plasma lipid concentrations of nephrotic rats with high and low plasma dextran levels*

	Dextran conc. > 2 g%	Dextran conc. < 1 g%	p†
Number of rats	10	10	
Triglycerides	0.95 ± 0.01‡	0.54 ± 0.01	<0.01
Total cholesterol	0.69 ± 0.02	0.35 ± 0.02	<0.01
Phospholipids	0.71 ± 0.02	0.36 ± 0.01	<0.01

* Calculated as the ratio of the change in concentration to the control concentration. Post-treatment concentrations were corrected for plasma volume change.
† Significance of the difference between the means of the two groups.
‡ Standard error of the mean.

From Table V it is evident that nephrotic animals that received dextran, PVP and γ-globulin had a slight increase in their mean corrected plasma albumin concentration during the experimental period, which in no instance was greater than 0.3 g per cent and which was not statistically significant in the dextran group. This slight rise was coupled with a decrease of less than 2 mg per hour in the mean proteinuria of those nephrotic rats receiving dextran and PVP. It should be noted that this increase in serum albumin concentration and decrease in proteinuria represent mean figures, and a number of rats (11 of 28 treated with dextran, 5 of 14 treated with PVP, and 4 of 12 treated with γ-globulin) showed no change, or the opposite change, in plasma albumin concentration concomitant with decreases in their hyperlipidemia. The administration of γ-globulin to nephrotic rats caused marked increases in their proteinuria, but essentially none of the γ-globulin administered to normal rats appeared in their urine during the treatment period. Though not statistically significant in these groups, it is of interest that the mean post-treatment albumin concentrations of all three groups of normal animals were higher than their

mean control values. This may represent overcorrection for plasma volume change.

In general, the higher the plasma concentration of the various macromolecular substances, the greater was the decrease in plasma lipid concentration. This relationship was noted following administration of dextran, PVP, γ-globulin and albumin, although there was considerable variation in the magnitude of the response of different animals to comparable plasma concentrations of these macromolecules. Table VI establishes the statistical validity of this observation for dextran-treated nephrotic rats of high and low plasma dextran concentrations.

Lipoprotein lipase assay. Figure 1 presents typical experiments in which plasma lipoprotein lipase activity is determined by its effect on clearing of a coconut oil suspension. By this method lipolytic activity is demonstrable in the plasma of both normal and nephrotic rats following intravenous administration of heparin. Note that when plasma obtained following the administration of heparin to normal rats is incubated with the coconut oil suspension, the initial phase of clearing is followed by a phase of increasing turbidity, as previously described by French, Robinson and Florey (20). Fortification of the clearing system with albumin enhances clearing and inhibits the return of turbidity, presumably owing to binding of the liberated fatty acids by albumin (21).

As is shown in Figure 1 we were unable to demonstrate that dextran, PVP, or γ-globulin, when administered intravenously in a variety of doses, shared heparin's ability to release lipoprotein lipase. Plasma obtained following a 3-day course of intraperitoneal injections of dextran or PVP showed no lipolytic activity. There was similarly no evidence that dextran, PVP or γ-globulin shared albumin's ability to enhance clearing by binding free fatty acids.

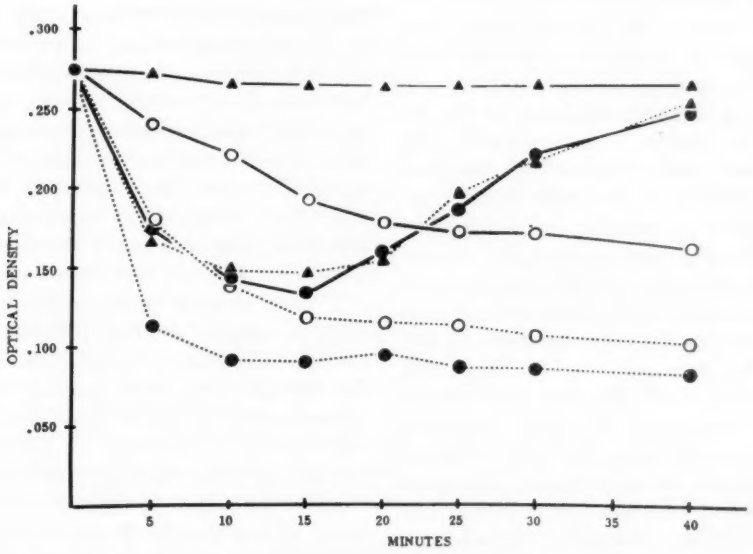

FIG. 1. CLEARING OF COCONUT OIL SUSPENSION BY RAT PLASMA (REPRESENTATIVE EXPERIMENTS). Plasma obtained from: ●——● Normal rat after intravenous heparin (see text for dosage). ●····● Normal rat after intravenous heparin; clearing system fortified with albumin. ▲····▲ Normal rat after intravenous heparin; clearing system fortified with dextran, polyvinlypyrrolidone (PVP) or bovine γ-globulin. ○——○ Nephrotic rat after intravenous heparin (fortification of clearing system with dextran, PVP or bovine γ-globulin yielded identical results). ○····○ Nephrotic rat after intravenous heparin; clearing system fortified with albumin. ▲——▲ Normal or nephrotic rat after intravenous dextran, PVP or bovine γ-globulin.

DISCUSSION

From the foregoing data it is evident that administration of dextran, PVP, bovine γ-globulin and bovine albumin can cause significant reductions in the hyperlipidemia of nephrotic rats. Though PVP apparently has some differential effect on the total cholesterol and phospholipid fractions, the general pattern of the plasma lipids following administration of these various substances to nephrotic rats is similar, making the hypothesis of a common mechanism for their action an appealing one.

It is impossible to delineate conclusively a mechanism for this lipid-lowering effect from the data presented in this report, but a number of possible mechanisms can be reasonably excluded on the basis of information available here. As judged by plasma albumin concentrations and proteinuria, there is no evidence of any change in the nephrotic status of these animals during the period of study. Similarly, the general appearance and behavior of the rats during the study did not suggest any general toxic effect of the administered substances, nor was there overt evidence of systemic effects from these substances in any but a small percentage of the animals. This observation is in agreement with those of Heymann and colleagues (5) who noted prompt re-elevation of serum lipids in their nephrotic rats following cessation of dextran therapy, and who demonstrated no azotemia following the use of this substance. A pilot experiment on the *in vitro* synthesis of lipoproteins by liver slices taken from nephrotic rats which had received dextran or PVP has been performed by the method of Radding, Bragdon and Steinberg (22). There was no gross impairment of hepatic lipoprotein synthesis in these rats compared with untreated nephrotic controls, although minor differences cannot be excluded from the data obtained (23).

In the experiments presented here we could find no evidence that acute or chronic administration of dextran, PVP or γ-globulin resulted in the release of lipoprotein lipase in the intact normal or nephrotic rat. This is of interest in that a number of anionic polysaccharides and polyelectrolytes, including heparin and dextran sulfate, do have this capability (24–27), and there is little doubt from the work of Opdyke and coworkers (28), Basu and Stewart (29), and Cohen and Tudhope (30) that chronic administration of substances which release lipoprotein lipase will significantly affect all of the fractions of blood lipids. Our inability to demonstrate lipoprotein lipase release by the compounds used in this study is in agreement with the observations of Stewart (31) who could demonstrate no effect of dextran comparable with that of heparin and dextran sulfate on increasing the electrophoretic mobility of lipoproteins, an effect ascribed by Gordon (32) to lipoprotein binding of the unesterified fatty acids released during lipolysis. As Gordon and co-workers (21) have shown, albumin potentiates lipoprotein lipase clearance by acting as a receptor for unesterified fatty acids. Under conditions in which we could demonstrate that albumin enhanced the lipoprotein lipase clearance reaction, we could demonstrate no comparable activity on the part of either dextran or PVP.

There are two possible mechanisms on which the present data give no information, but which would seem unlikely in the light of existing data. Both dextran and PVP are rapidly phagocytized by the reticuloendothelial system where they are stored for relatively long periods of time (33–35). The majority of existing evidence, however, indicates that serum cholesterol levels are raised rather than lowered by "blockade" of the reticuloendothelial system in rats and other animals (36–38). That metabolism of dextran or PVP could affect the plasma lipids in these animals seems unlikely. PVP is apparently not metabolized (33) and dextran is only slowly metabolized (39) by animals. Serum lipid changes following carbohydrate feeding in rats differ from those demonstrated here (40), making metabolism of glucose units derived from dextran an unappealing mechanism of action.

Some of those animals receiving the largest volumes by injection had diminished food intake. It is impossible from our data to exclude subtle dietary changes in the remaining animals, or to exclude covert systemic reactions to the administered substances. Preliminary data by one of the authors (41), however, seem to indicate that even after prolonged periods (7 to 10 days) of limited food intake, or a day of absolute fasting, reductions in the plasma lipids of nephrotic rats are significantly less than those shown in these data.

Although dextran, PVP, γ-globulin and albumin are of varying chemical structures, they share the ability to exert osmotic pressure in membrane systems. It is appealing to conclude, if only by exclusion, that this trait may cause at least a part of their lipid-reducing effects in nephrotic rats. The observations that similar patterns of lipid reduction result from the administration of these various substances, and that the magnitude of this reduction is in general directly proportional to the plasma concentration of the administered substance, are consistent with such a hypothesis. There are, of course, other conceivable mechanisms than those mentioned here, and as Baxter, Goodman and Havel (42) have pointed out, multiple factors undoubtedly influence nephrotic hyperlipidemia. Final elucidation of these various factors, as well as assessment of their relative importance, awaits further investigation.

SUMMARY

1. The administration of dextran, polyvinylpyrrolidone, bovine γ-globulin, and bovine albumin to nephrotic rats in doses sufficient to increase their plasma volume caused significant reductions in their plasma lipid concentrations.

2. Plasma triglyceride concentration decreased more than total cholesterol or phospholipid concentration during treatment with these substances.

3. Administration of these macromolecules to normal rats caused no significant fall in plasma lipid concentration, except in the case of polyvinylpyrrolidone which caused a decrease in total cholesterol and phospholipids. These reductions were proportionally less in normal rats than in nephrotic rats receiving polyvinylpyrrolidone.

4. In general, the magnitude of reduction of the plasma lipid concentrations was directly related to the plasma concentration of the macromolecular substance.

5. There was no significant change in the nephrotic state of the rats, as judged by plasma albumin concentration or proteinuria, adequate to explain the lipid changes.

6. There was no demonstrable evidence that any of these compounds released lipoprotein lipase, or enhanced clearing of plasma lipids by acting as acceptors for free fatty acids.

7. Some mechanisms for this lipid-lowering effect are discussed. The possibility that the osmotic activity of these macromolecular substances is causally related to the falls in lipid concentration is mentioned.

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SOME QUANTITATIVE ASPECTS OF THE BINDING OF QUINIDINE AND RELATED QUINOLINE COMPOUNDS BY HUMAN SERUM ALBUMIN *

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The interaction of quinidine with various body proteins may well be the fundamental process through which quinidine exerts its many effects. For example, quinidine binding with membrane lipoprotein may lead to decreased membrane permeability to passive ion movement. This permeability change has been held responsible for the quinidine-induced alterations in sodium and potassium exchange in cardiac muscle (1–4). Quinidine binding with critical enzyme proteins may be responsible for the alterations in intermediary metabolism which have been ascribed to quinidine (5). In our experience, glucose-6-phosphate dehydrogenase activity is reduced *in vitro* by quinidine. Finally, the known binding of quinidine to albumin may protect the cell against the action of quinidine by making less drug available at cellular sites. The nature of the interaction with protein of quinidine and of other compounds having a similar chemical structure seemed, therefore, to warrant an investigation.

Albumin is readily available in adequate amounts and in relative purity. As noted above, it is known to complex with quinidine. Further, the state of the quinidine-albumin interaction might be considered as a prototype of the "protective" form and perhaps characteristic of all forms of quinidine binding to protein. For these reasons our initial investigations were concerned with the reactions of quinidine and related quinoline compounds with albumin. Experiments were designed to permit determination of the stoichiometry, the association constant, and the energies of binding for the reactions; and to show the contributions to binding by the various reactive sites or areas in the quinidine molecule.

METHODS

A dialysis equilibrium technique was used to evaluate the *in vitro* binding of quinidine and of similar chemicals to human serum albumin.[1] A limited investigation of quinidine binding to human γ-globulin was carried out in the same manner.[2] A cellulose dialysis membrane [3] was prepared by an initial wash with 1 per cent sodium carbonate and a subsequent wash with distilled water. Twenty ml of 0.2 M phosphate buffer solution containing 250 mg of human serum albumin was placed in a dialysis bag. Fifty ml of the same buffer solution was placed around the bag in a plastic beaker. To this, external solution quinidine and other substances were added as desired. The beaker was then covered tightly with Saran wrap and shaken mechanically at 25° C until equilibrium had been achieved. In practice this was found to occur within 18 hours.

Quinidine may be considered structurally as 4-hydroxymethyl-6-methoxy-quinoline connected by the secondary alcohol bridge to a quinuclidine ring. Therefore, not only was the effect of quinidine binding to albumin studied but also the binding to albumin of 4-hydroxymethyl-6-methoxy-quinoline,[4] 6-methoxy-quinoline, 4-hydroxymethyl quinoline,[5] quinoline, and naphthalene. The hydrochloride salt was used in all cases except that of naphthalene. These compounds were used as a means of evaluating the contribution of binding of the various parts of the quinoline molecule. In addition, the competitive or inhibitory effects of all of the above substances, except naphthalene, on quinidine-albumin binding were studied during the presence both of quinidine and of one of the other compounds in the system. The part played in the binding reaction by the quinuclidine

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[1] Obtained from the Red Cross in solution form and from Pentex Co., Kankakee, Ill., in crystalline form. On paper electrophoresis both migrated as homogeneous peaks corresponding to albumin when barbital buffer (pH 8.6) and an ionic strength of 0.075 were used.

[2] Obtained from Sharp and Dohme, division of Merck, Inc., Philadelphia, Pa.

[3] A. H. Thomas Company, Philadelphia, Pa.

[4, 5] Synthesized with the generous aid of R. W. Greeff and Co., Nederlandsche Kininefabriek, Maarsen, Holland.

TABLE I

Binding of quinidine and related compounds to albumin

B*	C_q†	C_a‡		B	C_q	C_a	
	Quinidine				6-Methoxy-quinoline		
62 ± 3	3.0×10^{-6}	2×10^{-4}	n§ = 1.0	67 ± 5	6.0×10^{-6}	2×10^{-4}	$n = 6.1$
60 ± 3	6.0×10^{-6}	2×10^{-4}		66 ± 2	1.2×10^{-5}	2×10^{-4}	
55 ± 2	3.2×10^{-5}	2×10^{-4}		46 ± 2	8.0×10^{-6}	2×10^{-4}	$K_d = 6.0 \times 10^{-4}$
34 ± 4	2.0×10^{-4}	2×10^{-4}	K_d‖ = 1.3×10^{-4}	36 ± 2	1.6×10^{-3}	2×10^{-4}	$K_a = 1.7 \times 10^{3}$
28 ± 1	4.0×10^{-4}	2×10^{-4}					
19 ± 3	8.1×10^{-4}	2×10^{-4}			4-Hydroxymethyl-quinoline		
9 ± 2	2.2×10^{-3}	2×10^{-4}	K_a¶ = 7.7×10^{3}				
				42 ± 4	8.0×10^{-6}	2×10^{-4}	$n = 3.9$
	4-Hydroxymethyl-6-methoxy-quinoline			28 ± 2	9.0×10^{-4}	2×10^{-4}	
				18 ± 2	2.4×10^{-3}	2×10^{-4}	$K_d = 9.0 \times 10^{-4}$
73 ± 4	5.5×10^{-6}	2×10^{-4}	$n = 2.9$	12 ± 2	4.5×10^{-3}	2×10^{-4}	$K_a = 1.1 \times 10^{3}$
72 ± 4	1.0×10^{-5}	2×10^{-4}					
50 ± 2	3.6×10^{-4}	2×10^{-4}	$K_d = 2.0 \times 10^{-4}$		Quinoline		
38 ± 3	7.0×10^{-4}	2×10^{-4}					
25 ± 3	1.4×10^{-3}	2×10^{-4}	$K_a = 5.0 \times 10^{3}$	50 ± 2	8.0×10^{-6}	2×10^{-4}	$n = 8.1$
				48 ± 1	1.6 ± 10^{-5}	2×10^{-4}	
	Naphthalene			40 ± 3	8.0×10^{-4}	2×10^{-4}	$K_d = 1.7 \times 10^{-3}$
				34 ± 3	1.3×10^{-3}	2×10^{-4}	
66 ± 2	1.3×10^{-4}	2×10^{-4}	$n = 67.0$	19 ± 2	5.4×10^{-3}	2×10^{-4}	$K_a = 6.0 \times 10^{2}$
65 ± 2	2.5×10^{-4}	2×10^{-4}	$K_d = 6.6 \times 10^{-3}$				
			$K_a = 1.5 \times 10^{2}$				

* Per cent bound, with standard deviation.
† Free molar concentration of drug.
‡ Molar concentration of albumin.
§ Number of areas available for drug binding per mole of albumin.
‖ Dissociation constant of drug-albumin complex.
¶ Association constant of drug-albumin complex.

ring was evaluated in part through a study of the inhibitory effects of triethylene diamine (TED) and of benzyl alcohol on the quinidine-albumin interaction.

Quinidine concentration in protein and nonprotein solutions was determined in the Beckman DU spectrophotometer by the method of Josephson, Udenfriend and Brodie with ethylene dichloride as the extracting agent (6). The concentrations of 4-hydroxymethyl-6-methoxy-quinoline, 6-methoxy-quinoline, 4-hydroxymethyl-quinoline, quinoline, and naphthalene were determined in a similar fashion. However, the concentration of the first two compounds, as well as that of quinidine, was determined at 250 mμ, while that of the 4-hydroxymethyl-quinoline and quinoline was determined at 235 mμ and that of naphthalene at 220 mμ. In those experiments in which use was made of two compounds, both having an appreciable reading at the same wave length, the compounds were first separated on an acid alumina (Woelm) column with a solvent phase consisting of increasing (0.5 and 4.0 per cent) concentrations of ethanol in ethylene dichloride. Simultaneous recovery experiments were carried out following the addition of measured amounts of these various compounds both to the albumin and to the buffer solution of the dialysis system. If the recovery was not 100 ± 10 per cent, the experimental data were discarded. Recoveries from the column were 85 ± 5 per cent. From the concentrations of quinidine (or other compounds) measured on the two sides of the dialysis membrane, the free and bound fractions were calculated after a minor correction for the Donnan effect of protein.

The stoichiometric relationships and the dissociation constants of the drug-albumin complexes were evaluated as follows. The determinations of the bound fraction of the drug were made after the addition to the system of amounts of quinidine and/or quinoline compound varying from 100 to 50,000 μg. The formula reproduced by Goldstein (7) was solved to obtain values for the unknowns, K and n, which represent the dissociation constant and the stoichiometric relationships, respectively. The formula is $\beta = \frac{1}{1 + (K/nP) + (x/nP)}$ where β equals the fraction of bound to total drug, K equals the dissociation constant of the drug-protein complex, x equals the molar concentration of free drug, n equals the number of receptor locations in the albumin molecule for each drug molecule, and P is the molar concentration of the protein, here albumin. From the experimental data, solutions for the two unknowns were achieved either through the use of simultaneous equations or through the graphic plotting described by Goldstein (7). The association constant was derived as the reciprocal of the dissociation constant. The standard free energy of binding in kilogram-calories was calculated in the usual way by the product of the log of the association constant and 1.36. Each mean value noted in the results represents the average of at least 6 individual determinations, usually 10 to 12, and in some cases up to 30.

RESULTS

Stoichiometry and the dissociation constants of the quinidine-albumin complex. The stoichiometric relationship and the dissociation constants were obtained at pH values of 7.4, although they have been obtained at other pH values in experiments reported in another communication (8). The mean value for amount of drug bound at various free concentrations, the actual dissociation constants and the stoichiometric value are shown in Table I. The calculated results indicate that there is one receptor area for quinidine in each mole of albumin ($n = 1.04$). The dissociation constant is 1.3×10^{-4} at pH 7.4, although from other studies it is known to vary inversely with pH (8). Expressed as an association constant, this value becomes 7.7×10^{3} at pH 7.4. Thermodynamically, this gives a standard free energy of binding of about 5.3 kg-cal per mole of quinidine.

The binding of quinidine to γ-globulin. To the extent that these solutions resemble plasma, the K and n values obtained at pH 7.4 may be used to predict the protein-bound fraction of quinidine in the serum of patients given commonly employed doses of quinidine. With an average serum albumin concentration of 4 g per cent and a therapeutic serum-quinidine concentration of 5 mg per L (total concentration) the predicted value for the bound fraction would be about 80 per cent. A nearly identical value (0.78) has been reported to be the fraction of quinidine and also quinine normally bound in human serum (9, 10). The similarity of these figures suggested that quinidine is not bound to any significant extent by serum globulin and led us to test this hypothesis experimentally. As the data from the albumin experiments would suggest, the amount of quinidine bound to γ-globulin in a dialysis equilibrium system was not significantly different from zero.

The binding of 4-hydroxymethyl-6-methoxy-quinoline to albumin. The results of the binding studies carried out with this drug are shown in Table I. The calculated n indicates that there are three receptor locations for 4-hydroxymethyl-6-methoxy-quinoline in each mole of albumin. The dissociation constant is 2×10^{-4} and the association constant is 5×10^{3}. The calculated free energy of binding is 5.0 kg-cal, only slightly less than that calculated for quinidine-albumin binding. Consequently, by inference the quinuclidine ring contributes only slightly to the binding.

The binding of 6-methoxy-quinoline to albumin. Results for the interaction of 6-methoxy-quinoline with albumin are shown in Table I. The results indicate that 6 moles of this drug are bound by each interacting molecule of albumin. The dissociation constant is 6×10^{-4}. The association constant is therefore 1.7×10^{3}, and the free energy of binding 4.4 kg-cal.

The binding of 4-hydroxymethyl-quinoline to albumin. The results for the binding of this drug to albumin are shown in Table I. Calculations indicate four receptor locations for this drug in each molecule of albumin. The dissociation constant is 9×10^{-3}. This is equivalent to an association constant of 1.1×10^{3}. The free energy of binding is calculated at 4.1 kg-cal.

The binding of quinoline to albumin. The results for the binding of quinoline to albumin are shown in Table I. They indicate that there are eight receptor areas for quinoline in the albumin molecule. The dissociation constant is 1.7×10^{-3}, equivalent to an association constant of 6×10^{2}. The calculated standard free energy of binding is 3.5 kg-cal.

The binding of naphthalene to albumin. Because of the extremely low solubility of naphthalene in aqueous solutions it was impossible to vary the free concentration so as to determine with adequate accuracy either the number of albumin receptor areas for naphthalene or the dissociation constant for the reaction. From the figures for per cent naphthalene bound to albumin obtained under these restricting conditions an n of about 67 and a dissociation constant of about 6.6×10^{-3} were calculated. This is equivalent to an association constant of 1.5×10^{2}, indicating an approximate free energy value of 2.9 kg-cal. Despite the recognized inadequacy of the data and therefore the uncertainty of the extrapolations, they are presented as matters of interest in that *1*) the calculated free energy value is similar to that found for the association of albumin with non-polar substituents (personal communication from Dr. Fred Karush) and *2*) the free energy value is in general agreement with that predicted indirectly from our other experiments.

The effect of quinoline compounds in inhibiting quinidine-albumin binding. In order to show

that the several related quinoline compounds were actually combining at the same receptor location on the albumin molecule as quinidine, some studies were carried out with both quinidine and one quinoline compound present in the dialysis system. As described under Methods these substances were subsequently separated by column chromatography, and each compound was read at its proper spectral peak. On the basis of the previously determined K and n values for the quinoline compounds, an amount of each (one per experiment) was added to the system that would be expected to reduce quinidine binding very significantly under the existing experimental conditions, provided the two drugs competed for the same reaction area in the albumin molecule. From the measured concentrations of unbound quinidine and quinoline drug and from other pertinent data, a predicted value for the bound fraction of quinidine was calculated.[6] These values and the experimentally-obtained values were then compared with each other and with the predicted value for bound quinidine in the absence of a competing drug. The results are shown in Table II. The addition of sufficient 4-hydroxymethyl-6-methoxy-quinoline to the system to achieve a 6.4×10^{-4} M concentration of free drug reduced

TABLE II

Effect of quinoline compounds on quinidine-albumin binding

C_d*	C_q†	B_d‡	B_{eq}§	B_q‖	B_{eqs}¶	B_{eq}**
4-Hydroxymethyl-6-methoxy-quinoline						
6.4×10^{-4}	4.4×10^{-5}	40	41	27	23	54
6-Methoxy-quinoline						
2.1×10^{-3}	4.4×10^{-5}	25	31	25	32	54
4-Hydroxymethyl-quinoline						
4.4×10^{-3}	4.4×10^{-5}	10	13	22	30	54
Quinoline						
3.2×10^{-3}	4.4×10^{-5}	23	25	31	32	54

* Molar free concentration of quinoline compound found experimentally.
† Molar free concentration of quinidine found experimentally.
‡ Per cent of quinoline compound experimentally bound.
§ Per cent of quinoline compound expected bound on basis of K and n in Table I.
‖ Per cent of quinidine experimentally bound in presence of quinoline drug and albumin concentration of 2×10^{-4} M.
¶ Per cent of quinidine expected to be bound in presence of the amount of bound quinoline drug noted experimentally in column three (see footnote[6] in text).
** Per cent of quinidine predicted as bound if the quinoline drugs did not compete for albumin receptor or were not present in the solutions.

TABLE III

Effect of benzyl alcohol and triethylene diamine on quinidine-albumin binding

Benzyl alcohol				
C_{BA}*	C_q†	B_q‡	B_{eq}§	pH
2×10^{-3}	7.0×10^{-6}	65 ± 3	62	7.4
7×10^{-3}	7.2×10^{-6}	58 ± 2	62	7.4
2×10^{-2}	1.0×10^{-5}	23 ± 9	62	7.4
Triethylene diamine				
C_{TED}‖	C_q	B_q	B_{eq}	pH
1	7.0×10^{-6}	64 ± 2	90	10.0
1	8.0×10^{-6}	58 ± 4	62	7.4

* Molar concentration of benzyl alcohol.
† Molar free concentration of quinidine.
‡ Per cent of quinidine bound, with standard deviation.
§ Per cent of expected bound quinidine based on values from Table I.
‖ Molar concentration of triethylene diamine.

[6] Preliminary assumptions were made that not only the quinoline compound and quinidine were competing simultaneously for one reaction area in albumin but also that the dissociation constants obtained experimentally for the quinoline compound-albumin interactions were applicable to interaction at this one location. The predicted value for the fraction of quinidine bound in the presence of other drugs was then obtained as follows. The fraction of quinoline (competing) drug found to be bound experimentally (column 3, Table II) gives an equation relating the concentration of bound drug to the total drug concentration in the albumin solution. This relationship was used with a second equation relating the measured concentration of unbound or free quinoline to the concentration of bound drug and to the total concentration. In the case of 4-hydroxymethyl-6-methoxy-quinoline, 0.40 total equals bound, and total equals 6.4×10^{-4} plus bound. Solution of the simultaneous equations gives a value for the concentration of drug bound to albumin. This concentration divided by the number of protein receptor areas for the drug gives the concentration of drug bound at the one location at which quinidine may attach or compete. This result then presumably gives the concentration of protein receptor areas covered by competing drug and not available for quinidine binding—$4.27 \times 10^{-4}/2.9$ (B_c/n) equals 1.47×10^{-4}. The difference between this value and the total available areas (P_c or $2 \times 10^{-4} \times n$ or 1) gives the concentration of protein receptor locations still available for quinidine-albumin interaction, ($2 \times 10^{-4} - 1.47 \times 10^{-4}$ equals 0.53×10^{-4}). By substitution of this value, along with the appropriate K, n, and F_c for quinidine, in the Goldstein equation a predicted value for the bound fraction of quinidine is obtained (column 6, Table II).

the percentage of quinidine bound (unbound quinidine concentration of 4.4×10^{-5} M) from 54 to 27, close to the predicted value of 23 per cent. With a concentration of free 6-methoxyquinoline of 2.1×10^{-3} nearly the same result was achieved, a reduction in the quinidine bound from 54 to 25 per cent. This may be compared with the predicted result of 32 per cent bound. A reduction of the bound fraction of quinidine from 54 to 22 per cent was achieved with a 4.4×10^{-3} M concentration of 4 hydroxymethyl quinoline. The 22 per cent figure is similar to the predicted value of 30 per cent. A free quinoline concentration of 3.2×10^{-3} M reduced the fraction of quinidine bound to albumin from 54 to 31 per cent, almost exactly as predicted. The data consequently provide strong evidence that there is competition between quinidine and the related compounds tested for one specific reactive area in albumin.

The effects of triethylene diamine and benzyl alcohol on quinidine binding with albumin. An attempt to learn something further about the importance of the quinuclidine ring and the alcoholic OH site to the binding reaction was carried out through the addition of varying amounts of TED or of benzyl alcohol along with quinidine to the dialysis system. Addition of benzyl alcohol, which presumably might compete for an albumin site with the combined quinuclidine ring and adjacent alcohol fragment of quinidine, led to the results indicated in Table III. In the presence of free quinidine, concentrations of slightly less than 1×10^{-5} M benzyl alcohol in 2×10^{-3} M concentrations had little effect on binding. In the presence of a 7×10^{-3} M alcohol concentration, bound quinidine was reduced slightly from the 62 per cent expected if no alcohol were present to 58 per cent. With concentrations of benzyl alcohol at 2×10^{-2} M the inhibition of quinidine-albumin binding was very marked. An average of only 23 per cent was calculated for the bound fraction as compared with an expected 62 per cent. This is compatible with a contribution by the quinuclidine ring and the secondary alcohol of something like 1.5 kg-cal to the total energy of binding, a value close to that predicted from the previously noted experimental results. On the other hand, TED which presumably is similar to the basic quinuclidine structure, failed to reduce appreciably quinidine binding at pH 7.4 even when present in concentrations as great as 1 M. Only a slight reduction from an expected value of 62 per cent to a calculated value of 58 per cent was found with 1 M concentrations. Since the two pK's of triethylene diamine are 5 and 8.4, at pH 7.4 one of the nitrogens is mainly positively charged, as is the nitrogen in the quinuclidine ring (pK, 8.6). Therefore, further evidence is adduced that the quinuclidine ring per se does not seem to play an important part in the binding of quinidine to albumin, at least when the nitrogen group is positively charged, but that the alcoholic bridge fragment contributes something like 1 kg-cal to the reaction.

In view of these conclusions, we were somewhat surprised to find that in the presence of carbonate buffer at pH 10, the same concentration of TED reduced the binding to 64 per cent from an expected 90 per cent. This significant reduction in the binding, however, occurs at a pH at which the two nitrogens in the triethylene diamine and the nitrogen of the quinuclidine ring of quinidine are essentially all noncharged. We have found that at this pH the increased binding, found normally, relates mainly to an increase in the number of quinidine receptor areas in albumin from 1 to 3 (8), possibly because of the loss of the polar effect of the positively charged nitrogen. This increased n value (3.0) is the same as that calculated for 4-hydroxymethyl-6-methoxy-quinoline. It is suggested that neutral TED in 1 M concentration may compete with neutral quinidine for these additional two albumin binding areas.

DISCUSSION

These data reveal a moderately large (K_a 7.7×10^3) association constant for the quinidine-albumin reaction, and an uncommon, one-for-one molar interaction—i.e., one receptor area for quinidine attachment in each albumin molecule. The basic quinoline ring structure apparently contributes a major portion of the binding energy, about 2.9 out of 5.3 kg-cal. The neutral nitrogen of the quinoline ring (pK, 4.0), the methoxy group, and the secondary bridging alcohol group also contribute to the energy of binding and therefore presume to be sites of interaction with

the albumin molecule.[7] The specificity of the quinidine-albumin reaction is contributed to by all the three reactive groups as is evident by the increasing number of binding areas in albumin involved and the decreasing association constant values resulting as these groups are deleted from the molecule. Either hydrogen bonding or Van der Waals forces could account for these interactions. Formation of hydrogen bonds seems more probable in view of the virtually identical binding energies (0.5 to 1.0 kg-cal) concerned with each of the three reactive sites, and particularly because the experimental data appear to show that the energy of binding is the same for all albumin locations involved in binding both quinidine and related quinoline compounds. Equal Van der Waals interactions in all cases would imply nearly identical spatial complementarity of albumin and drug at the critical areas. It is improbable that the eight areas concerned in albumin would have the required structural identity. On the other hand, albumin sites at which hydrogen bond linkage to the quinoline nitrogen, the methoxy, and the hydroxymethyl groups of quinidine might occur have not been identified. Our recent unreported studies are compatible with the concept that the quinuclidine nitrogen forms a hydrogen bond with an imidazole nitrogen in a histidine residue of albumin. The methoxy group may form a hydrogen bond with a hydroxyl group in serine, and the suggestion has been made that a similar reaction may link the secondary alcohol to a hydroxyl group in tyrosine (personal communication from Dr. I. M. Klotz).

At pH 7.4, the quinuclidine ring does not seem to play a very great part in the binding of quinidine to albumin. Its most important role may relate to the action of the charged nitrogen in preventing binding at more than one reactive area in albumin. This role is suggested *1*) by the fact that the simpler compound without the quinuclidine ring—4-hydroxymethyl-6-methoxy-quinoline—appears to react at three binding areas in albumin and *2*) by the fact that when the positive nitrogen in the quinuclidine ring becomes neutral with increasing pH, the number of receptor areas for quinidine in albumin is also three. In brief, the uncharged form of quinidine may be able to attach itself at two additional areas in albumin where the charged form is unable to penetrate or attach. While this hypothesis may appear more attractive, an additional consideration is deserving of mention—competition for quinidine between the water and protein phases. Water solubility of quinidine varies markedly with pH in an inverse fashion. This relationship might be anticipated if the charged nitrogen groups (pK's of 4.0 and 8.6) were undergoing polar interactions with water. At a pH of 10, quinidine is nearly all in the uncharged form, quinidine solubility is slight, and with increasing pH, it rapidly becomes negligible. With decreasing pH below 7, quinidine solubility increases markedly, the acidic salts—quinidine hydrochloride and quinidine sulfate—being the compounds commonly used to prepare concentrated aqueous solutions of quinidine. Quinidine's high aqueous solubility at low pH, reflecting predominance of the most polar forms of the molecule, might be partially responsible for the reciprocally low degree of attachment to protein we have observed in unreported studies. In contrast, low aqueous solubility of the neutral form of the drug at high pH may account partially for the marked drug affinity for albumin noted under these conditions. To the extent that this process is operative, a plot of quinidine binding by albumin against pH should resemble a quinidine acid titration curve. Unfortunately, the former plot as obtained experimentally is more complex than the

[7] The term *site* as commonly used in characterizing drug-protein interaction has at least two connotations and needs to be defined for purposes of the present discussion. It has been used to refer to the point of molecular interaction between a reactive group or atom in the drug and a corresponding group in an amino acid of protein. Electrostatic interaction between a positively charged nitrogen atom in a drug and a negatively charged hydroxyl group in protein is one such example. We have reserved *site* for use in describing this form of interaction. *Site* or *receptor site* has also been commonly used to refer to the general area at which a drug is attached to a protein, irrespective of whether one or many drug-protein reactive group interactions take place, or whether, in our terminology, one or many sites of interaction are involved. Thus, in common parlance n is defined as the number of drug receptor sites per molecule of protein. In order to avoid needless confusion between the former (single) and the latter (in this case, multiple interaction), we have arbitrarily substituted the terms *area* or *location* for use in the second connotation. We have defined n as the number of receptor locations (or areas) for drug attachment to the albumin molecule.

latter. Interpretation is not so simple as proposed and the issue remains unresolved.

The similarity of association constants for the quinidine-albumin and the (4-hydroxymethyl-6-methoxy-quinoline)-albumin reactions, and the finding of a receptor area in albumin available to both drugs led us to test whether these drugs have any common cardiovascular effects (11). The substituted quinoline does have a peripheral vasodilation action like that of quinidine. The hypotension produced by both drugs is similarily reversed by sympathomimetic amines. Finally the effective "hypotensive" dose of 4-hydroxymethyl-6-methoxy-quinoline is three or four times that of quinidine, a relationship predictable by translation of the relative quantitative aspects of the drugs binding with albumin to their binding via a similar hypothetical receptor in arteriolar protein. A comparison of the action of the two drugs on the heart gives a different picture. The substituted quinoline compound possesses minimal antiarrythmic and apparently none of the usual myocardial depressant properties of quinidine. The indirect implication is that the pharmacologic effects of quinidine on the heart are dependent on the presence of the quinuclidine ring. From these considerations two questions are posed, neither of which is currently answerable except in an indirect and speculative fashion.

The first question is concerned with the extent to which the characteristics of quinidine-albumin interaction can be considered to reflect the nature of quinidine binding by cell protein. Studies such as those of Wegria and Boyle (12) tell us simply that there must be a considerable amount of cellular quinidine binding to protein. We are aware of only one quantitative study concerning quinidine binding to isolated purified cell protein. This is one of our unreported studies involving the use of *Escherichia coli* endotoxin, a material thought to be membrane lipoprotein although perhaps modified in preparation. From the results of that study we calculated that the number of receptor areas for quinidine per molecule of endotoxin (based on an estimated 10^6 molecular weight of endotoxin) is about 1.5. While this n value is similar to that for the reaction between quinidine and albumin, the association constant at pH 7.4 is approximately 100-fold greater, being 8.0×10^5. It may well turn out that quinidine is highly associated with certain fractions of cell protein and negligibly associated with others. Our findings of an apparently rather specific interaction between quinidine and albumin and a negligible or absent one between quinidine and γ-globin makes this speculation plausible.

The second question is: Why should the presence of the quinuclidine ring be critical to the cardiac but not to the peripheral vascular action of quinidine? The cinchona alkaloids have been known for many years to have the properties of a surface film agent. Accordingly, the cardiac cell surface has been proposed as a critical site with respect to quinidine action. The results indicating a quinidine-induced alteration in ionic transfer rates and ion distribution are compatible with this concept (1–4). Other surface active agents derive their activity from the hydrophilic nature of one portion of the molecule and a nonaqueous phase affinity of another. The substance is thus molecularly oriented so as to link aqueous and nonaqueous phases. From the observed characteristics of quinidine-albumin binding and of quinidine solubility in water, it appears that under appropriate circumstances quinidine can play this role. The quinoline portion of the molecule is albuminophilic and binds to protein, while the charged quinuclidine ring is hydrophilic and interacts with water. If this kind of reaction were required at the cell membrane interfaces in order to bring about the myocardial effects of quinidine, the necessary presence of the charged quinuclidine ring would be explained. We should therefore like to suggest the possibility that the cardiac effects of quinidine can be at least in part explained by its molecular orientation at cell interfaces.

SUMMARY

The quantitative aspects of quinidine binding to albumin were investigated in an *in vitro* dialysis equilibrium system. Similar investigations were made using related compounds—4-hydroxymethyl-6-methoxy-quinoline, 6-methoxy-quinoline, 4-hydroxymethyl-quinoline, quinoline, and naphthalene. The inhibitory effects on quinidine-albumin binding of these latter drugs, save naphthalene,

and the effects of benzyl alcohol and triethylene diamine were evaluated. Quinidine-γ-globulin binding was also evaluated and found to be negligible.

The quinidine-albumin reaction is characterized by the presence of one receptor area for quinidine per protein molecule and a moderately large association constant (7.7×10^3). The interaction of albumin and the quinoline compounds related to quinidine is characterized by an increasing number of available binding sites in albumin and decreasing association constants as the chemically active sites in the quinoline molecule are eliminated. All the quinoline drugs tested appear to compete with quinidine for a presumed common receptor area in albumin. This competition occurs in a manner which is at least semiquantitatively predictable from quinoline relationships with albumin, relative to those of quinidine with albumin.

Quinidine binding by albumin seems to depend upon protein interaction with the basic quinoline ring and a more or less equal interaction with the quinoline nitrogen, the methoxy, and the secondary alcohol groups. The last three groups probably interact with protein sites as a result of hydrogen bond formation. Minor interaction of the quinuclidine ring structure and albumin could not be excluded.

Observations on the similarities of 4-hydroxymethyl-6-methoxy-quinoline and of quinidine binding to albumin, on their similarities of structure, and on their comparative effects on the cardiovascular system may be considered in agreement with two hypotheses. Quinidine-albumin binding reflects the nature of the combination of quinidine with a "critical" receptor in the protein of arteriolar smooth muscle. Cardiac action of quinidine may result from a special orientation of the molecule at interfaces, with binding of the quinoline ring to interface lipoprotein and of the charged quinuclidine ring to water.

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OCCURRENCE OF COMPLEMENT-FIXING ANTIBODIES REACTIVE WITH NORMAL TISSUE CONSTITUENTS IN NORMAL AND DISEASE STATES *

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Serum substances that react with mammalian tissues, present in individuals with certain acute and chronic diseases, have been the object of several recent reviews (2–4). In addition, complement-fixing substances against human tissue have been demonstrated recently in patients with acute viral infections and systemic lupus erythematosus (5). The question of whether these substances are true antibodies, formed as a result of antigenic stimulation, has often been raised. As far as they have been characterized, they possess the attributes of typical antibodies formed in response to injections of foreign materials: relative specificity, occurrence in the globulin fraction of serum, ability to fix complement, and relative heat stability. It would seem reasonable, therefore, to regard them as antibodies.

The implied detrimental effect of an immunological response to one's own antigens and the subsequent production of tissue antibodies was dramatized by Ehrlich with the expression *horror autotoxicus*. Yet Kidd and Friedewald (6) have demonstrated the existence of tissue antibodies in the sera of normal rabbits that fix complement with constituents of normal rabbit tissue cells. The present work has considered the incidence of these natural antibodies in other species in addition to the rabbit and their possible reactivity against calf thymus as well as rabbit tissue. In addition, the level of these antibodies in human disease and in animals subjected to various experimental conditions has been determined. Finally, certain properties of tissue antibodies have been investigated.

* A preliminary report of this work was presented at the 1960 meeting of the American Association of Immunologists (1).

MATERIALS AND METHODS

Antigens. Tissue homogenates were prepared from calf thymus and from rabbit liver as described by Gajdusek (5) for human tissues. The stock antigen preparations used contained about 50 and 100 mg protein per ml, respectively, and were free of detectable DNA (Dische test). For the performance of the test, each antigen was diluted 1:3, 1:9, and 1:27, and 0.3 ml of these dilutions was used. In each instance this represented 5 mg or less of antigen. None of the preparations was anticomplementary under the conditions of the test, and there was no apparent loss of serologic activity during storage for 4 weeks at − 20° C.

Hemolytic system. The test procedure was a modification of a precise method developed by the Department of Serology, Walter Reed Army Institute of Research (7). The hemolytic system consisted of a suspension of washed sheep erythrocytes, spectrophotometrically standardized to contain 5×10^8 cells per ml, and sensitized with an optimal concentration of hemolysin. Guinea pig complement was titrated by a precise technique in which the 50 per cent hemolytic unit ($C'H_{50}$) was estimated by a plot of the logarithm of the complement volume against the percentage hemolysis converted to probits. The reagent diluent was 0.15 M salt solution of pH 7.3 to 7.4 containing Mg^{++} and Ca^{++} ions in concentrations optimal for hemolysis buffered with triethanolamine.

Test procedure. Each serum was inactivated for 30 minutes at 56° C and serially diluted in the diluent before testing. For the test proper, 0.3 ml of serum, complement containing 5 $C'H_{50}$ units, and diluted antigen were combined and incubated for 16 to 18 hours at 4° C. After the incubation period, 0.6 ml of sensitized erythrocytes was added, and 30 minutes in the water bath at 37° C was allowed for hemolysis. The tests were then read by comparison with color standards simulating different percentages of hemolysis. The titer for any serum was considered as the reciprocal of the highest dilution giving not greater than 50 per cent hemolysis with any of the employed dilutions of antigen.

RESULTS

Tests with normal sera of different species. The occurrence of complement-fixing (CF) tis-

TABLE I

The reactivity of sera of different normal animals against tissue antigens

Species	Antigen	No. tested	CF titers (reciprocals) Nonreactive at 1:3	3	9	27	81
Man	Rabbit liver	68	18	37	12	1	0
	Calf thymus	55	33	16	5	1	0
Chimpanzee	Rabbit liver	2	0	0	2	0	0
	Calf thymus	2	0	2	0	0	0
Monkey	Rabbit liver	2	0	0	1	1	0
	Calf thymus	2	0	0	1	1	0
Rabbit	Rabbit liver	6	1	3	2	0	0
	Calf thymus	15	1	3	5	5	1
Guinea pig	Rabbit liver	19	19	0	0	0	0
	Calf thymus	19	19	0	0	0	0
Mouse	Rabbit liver	2 pools*	2	0	0	0	0
	Calf thymus	2 pools*	2	0	0	0	0
Rat	Rabbit liver	3 pools*	1	0	2	0	0
	Calf thymus	5 pools*	1	4	0	0	0

* Pools of equal volumes of 5 individual sera.

sue antibodies in the sera of normal rabbits was confirmed. The sera of 19 normal guinea pigs, however, were found to be nonreactive with both rabbit liver and calf thymus. Sera of healthy young male adults, candidates for admission to the United States Military Academy at West Point, were variable in their reactivity. These results, together with those obtained in testing several other animal species, are tabulated in Table I.

Tests with sera of laboratory animals. Rabbits inoculated intratesticularly with *Treponema pallidum* developed high antibody titers against the cardiolipin antigens used in the serodiagnosis of syphilis, but unlike human patients, showed scarcely any rise in titer against calf thymus or rabbit liver saline extract antigens (Table II). On the other hand, rabbits infected with *Trypanosoma gambiense* or *T. rhodesiense* developed significant antibody increases against saline extract antigens, but essentially no rise in Wassermann

TABLE II

Response of a rabbit to inoculation with T. pallidum

Serum	Wassermann antibody (cardiolipin microflocculation) titer (reciprocal)	CF titer (reciprocal) against calf thymus
Preinoculation	Nonreactive	27
Day, postinoculation		
5	2	27
10	64	27
17	64	27
24	32	27
31	8	27
45	16	27

antibody (Table III). One notes in Table III a 27-fold rise in titer against rabbit liver and at least a 3-fold rise against calf thymus. The decline in C′ level from 34 to 19 may be related to the development of tissue antibody and consequent *in vivo* fixation by antigen-antibody complex, but the titer of 40 on the thirteenth postinoculation day is not easily explained.

The development of tissue antibodies does not by any means, however, invariably follow microbial infection or the injection of microbial products. Guinea pigs infected with lethal doses of the Bitterroot strain of Rocky Mountain spotted fever, carbon tetrachloride-treated guinea pigs infected with *Shigella* organisms, and chimpanzees orally infected with *Salmonella typhosa* have not developed elevated levels of these antibodies. Animals subjected to other experimental conditions also did not develop elevated levels of tissue antibodies. These included severely burned rats, guinea pigs subjected to X-irradiation (400 r), mice subjected to X-irradiation (600 r), and rabbits injected three times with a solution containing poliomyelitis, epidemic and endemic typhus vaccines or tetanus-diphtheria toxoids, plague, and typhoid and paratyphoid A and B vaccines.

TABLE III

Response of rabbit to inoculation with T. gambiense

Serum	CF titer (reciprocal) against calf thymus	CF titer (reciprocal) against rabbit liver	C′ titer ($C'H_{50}$/ml)*	Cardiolipin microflocculation
Preinoc.	27	3	34	Nonreactive
Day, postinoc.				
13	81 or >	81	40	Nonreactive
28	81	81	19	Trace reaction (titer < 1)

* Represents complement titer in 50% hemolytic units per ml of serum.

TABLE IV

Comparison of titers (reciprocals) of 20 sera of patients with rheumatoid arthritis in the bentonite flocculation test and in the complement fixation procedure against calf thymus

Bentonite flocculation titer	No. of sera tested	CF against calf thymus			
		Nonreactive at 1:3	3	9	27
16	2	1	1	0	0
32	3	3	0	0	0
64	3	1	2	0	0
128	2	2	0	0	0
256 or >	13	9	1	3	0
Total	23	16	4	3	0

Tests with human sera; connective tissue diseases. Sera which were reactive in the bentonite flocculation test (8) for rheumatoid factor, from patients with rheumatoid arthritis, were tested with calf thymus antigen in CF tests. The results given in Table IV indicate that the majority of sera (13 out of 20) from such patients do not have detectable tissue antibody levels, irrespective of rheumatoid factor levels. As was observed in Table I, similar results were obtained with sera from normal healthy individuals. A smaller number of the rheumatoid arthritic sera similarly tested against rabbit liver were mostly nonreactive. Whereas sera of patients with rheumatoid arthritis generally failed to react with tissue antigens, 32 of 33 sera from patients with systemic lupus erythematosus reacted against the calf thymus antigen and to a much higher average titer; the single exception was a patient in a state of remission (Table V). Sixteen of these sera were also tested against rabbit liver and in general yielded comparable but higher titers than those obtained in parallel tests with calf thymus antigen.

Lues and biological false positive (BFP) reactors. An appreciable percentage of sera from patients with luetic infection, verified by clinical status and positive treponemal immobilization tests, reacted against both tissue antigens at elevated levels. Twelve of 26 sera, for example, from the luetic patients reacted with titers of 1:9 or greater against rabbit liver. An association was observed, moreover, between the Wassermann antibody titer given in the cardiolipin microflocculation test of the United States Army (9) and the complement-fixation titers against rabbit liver (Table VI). In addition, tests against calf thymus were performed with eight of the sera; three of these specimens gave slide flocculation titers of 32 or greater, and among these were the only two sera with elevated levels of complement-fixing antibodies against calf thymus. Wassermann antibody was shown not to be identical with rabbit liver and calf thymus tissue antibodies, however, by absorption experiments. Absorption of Wassermann antibody from luetic sera by cardiolipin floccules resulted in relatively slight losses of the tissue antibodies. In contrast to the association

TABLE V

Complement fixation titers (reciprocals) against calf thymus of 33 sera of patients with systemic lupus erythematosus

Total no. tested	Nonreactive at 1:3	CF titers		
		3	9	26 or >
33	1	8	7	17

TABLE VI

Comparison of titers (reciprocals) of Wassermann antibody obtained with the cardiolipin microflocculation test and of complement-fixing antibodies against rabbit liver in 26 sera of luetic patients

Micro-flocculation titers	No. tested	CF against rabbit liver			
		Nonreactive at 1:3	3	9	27
<1	2	1	1	0	0
1	5	2	1	2	0
2	4	2	2	0	0
4	1	1	0	0	0
8	2	1	1	0	0
16	3	0	1	2	0
32 or >	9	0	1	4	4

TABLE VII

Comparison of titers (reciprocals) for Wassermann antibody with the cardiolipin microflocculation test and calf thymus antibodies in sera of individuals with biological false positive reactions of lues

Cardiolipin microflocculation		No. tested	Titer against calf thymus			
			Nonreactive at 1:3	3	9	27 or >
Weak reaction titer	<1	23	9	11	1	2
Reaction titer	1	24	12	7	2	3
	2	11	6	3	0	2
	4	5	1	1	0	3
	8	5	2	1	2	0
	16	2	0	1	0	1
	32	1	0	0	1	0
Total		71	30	24	6	11

TABLE VIII

Tissue antibody levels in a patient with Rocky Mountain spotted fever complicated by a paracolon infection

Days after onset of illness*	CF titers (reciprocals) against rabbit liver	CF titers (reciprocals) against calf thymus
4	Neg. at 1:3	Neg. at 1:3
13	9	9
20	27 or >	27
50	9	3

* Expired on Day 58.

observed between the Wassermann and tissue antibody levels with the sera of luetic patients, the sera of 71 individuals exhibiting BFP reactions—i.e., reactive for Wassermann antibody but nonreactive in the treponemal immobilization test and without clinical evidence or a history indicative of syphilis—did not reveal a similar correlation. The most extensive testing of these sera was performed with calf thymus antigen; the findings are summarized in Table VII. The lack of correlation is observed readily, for example, with the 11 sera giving a titer of 27 or greater against calf thymus in which the cardiolipin microflocculation titers ranged from less than 1 to 16. Entirely comparable results were obtained with 50 of these sera tested similarly against rabbit liver antigen.

Other infectious diseases. Sera from 15 human cases of poliomyelitis, 8 cases of mumps, and 8 cases of pericarditis of unknown etiology, obtained at various disease stages, were nonreactive or within normal levels with both antigens. However, in a patient with Rocky Mountain spotted fever complicated by paracolon infection, a marked rise and decline, prior to his death, occurred in tissue antibody levels during course of the disease (Table VIII).

Hypocomplementemia. Apparently unrelated to any disease process, slightly elevated tissue antibody levels (titer 1:9 against calf thymus, and 1:9 or greater against rabbit liver) have been found in a healthy individual who had had extremely low levels of hemolytic complement for several years (10). The complement level in this individual was about one-tenth that of normal, and the deficiency was in the second component.

Antibody characteristics. The question was raised as to whether the tissue antibodies in patients with connective tissue diseases and in animals with trypanosomal infection were similar to those found in normal humans and rabbits. Kidd and Friedewald (6) observed that essentially all of the antigen in rabbit liver reactive with normal rabbit serum was sedimented at 30,000 rpm, comparatively little or none of it remaining in the supernatant fluids. Similar findings were ob-

TABLE IX

Reactions of different sera with centrifuged extracts of normal rabbit liver *

Serum	Antigen	Antigen dilution 1:9†	1:27	1:81	1:243	1:729	1:2187
Normal rabbit 121757-2, 1:3 diln.	Whole extract	20	0	0	0	0	15
	Supernatant	15	10	60	85	100	100
	Sediment	5	0	0	0	10	35
Normal human 30960-7, 1:3 diln.	Whole extract	45	90	100	100	100	100
	Supernatant	100	100	100	100	100	100
	Sediment	20	85	100	100	100	100
Human lupus erythematosus‡ 22960-1, 1:9 diln.	Whole extract	10	0	0	95	100	100
	Supernatant	10	20	100	100	100	100
	Sediment	0	0	35	95	100	100
Rocky Mountain spotted fever patient, 82659-1	Whole extract	20	0	0	30	100	100
	Supernatant	20	20	100	100	100	100
	Sediment	0	0	0	40	100	100
Rabbit anti-*T. gambiense*, 11558-2	Whole extract	15	0	0	0	5	60
	Supernatant	10	0	80	100	100	100
	Sediment	5	0	0	0	15	60

* For the preparation of the extracts, the whole extract was centrifuged at 100,000 G for 1 hour. The sediment was suspended with saline to the original volume of the extract. The numbers represent percentages of hemolysis.

† With the exception of the reactions with serum 30960-7, the lack of complete fixation with the 1:9 antigen dilution probably was caused by an antigen prozone.

‡ Two other sera of patients with lupus erythematosus reacted similarly.

TABLE X

The heat lability of tissue antibodies against rabbit liver in normal and pathological sera

Temp, ° C*	Serum	CF titers (reciprocals)
60	Normal rabbit	9
65	Normal rabbit	3
60	Rabbit anti-*T. gambiense*	81 or >
65	Rabbit anti-*T. gambiense*	9
56	Normal human	3
65	Normal human	Nonreactive at 1:3 dilution
56	Human lupus erythematosus	81
65	Human lupus erythematosus	9
56	Human Rocky Mountain spotted fever	27
65	Human Rocky Mountain spotted fever	9

* Prior to testing, all sera were heated at the temperatures indicated for 30 minutes. Rabbit sera are ordinarily heated at 60° instead of 56° to minimize their anticomplementary properties.

served in testing certain human sera, including sera from normal individuals, several patients with systemic lupus erythematosus, and one with Rocky Mountain spotted fever (Table IX). The heat lability of normal rabbit tissue antibody was paralleled also by these human sera (Table X).

DISCUSSION

The existence of natural antibodies in rabbits (6) that react *in vitro* with constituents of normal rabbit tissue cells has been confirmed by the findings in this study. Like other so-called natural antibodies, the incidence of normal tissue antibodies depends upon the species. All of the guinea pig and mouse sera examined were nonreactive against either rabbit liver or calf thymus. In man, on the other hand, a rather high incidence of antibodies against these antigens was found: 74 per cent of sera of normal individuals was reactive against rabbit liver and 40 per cent against calf thymus. A higher incidence of reactivity and higher mean titers were found with the rabbit sera.

Certain characteristics of the tissue antibodies of both the natural and the "acquired" variety, found in the sera of humans with systemic lupus erythematosus and in the sera of humans and animals with infecting diseases, are very similar. They have comparable heat stabilities, and both types react with the relatively easily sedimentable constituents of the tissue extracts. In view of the possible similarity of normal tissue antibodies and those tissue antibodies present at elevated levels in sera of certain patients, it follows that the processes leading to normal levels of tissue antibodies may be accentuated for unknown reasons in certain patients, or conceivably, but less likely, that the elimination of these antibodies is delayed in those patients. The origin of these tissue antibodies has intrigued immunologists for a long time. Their existence is even more puzzling today because of very extensive investigations which have placed the principle of immunological tolerance on a firm basis. According to this principle, if a foreign antigen is introduced into an animal sufficiently early, the antibody-synthesizing mechanism will accept its persistence or later introductions of it without antibody response. Thus, the peculiar mystery of the origin of tissue antibody arises from the presence of tissue antigens as early as conception. Despite this theoretical objection to their presence, two distinct mechanisms have been postulated to account for the formation of tissue antibodies. In the first of these, normal tissue cells are assumed to be injured and altered by harmful agents and thereby rendered antigenic. Microbial agents, cold, heat, and X-rays have been postulated as irritants for the conversion of normal tissue constituents into antigens (11). Our results have failed, however, to demonstrate the occurrence of such a process in experimental animals subjected to extensive burns or X-rays, but have shown its occurrence as a result of microbial invasion. The resulting reaction of a host to its own tissues has been termed *autosensitization*. Although the antigens used in the present study were usually not derived from the species whose serum was being studied, comparable results with homologous antigens have been ob-

tained in studies reported by others (12) and in other studies of our own. In our experience, most reactive human sera have shown a similar spectrum of activity with antigens derived from human, rabbit, and bovine livers. Thus, species specificity seems not to be generally involved. Organ specificity is likewise apparently lacking and tissue antibodies are generally capable of reacting with liver, kidney, and thymus. Thus, similar antigenic determinants seem to be predominantly involved in the serological reactivity of tissue extracts, although different tissues are undoubtedly not antigenically identical. Organ specific antibodies have been reported in patients with thyroiditis (13) and pancreatitis (14). The second possibility is that the antigenic components of a microbial agent may give rise to the formation of antibodies cross reactive with tissue constituents. This latter process, designated *heterosensitization,* has been invoked recently in attempts to explain the presence of deoxyribonucleoprotein antibodies in patients with systemic lupus erythematosus. These antibodies are lacking in species specificity and, conceivably, bacterial or viral nucleoprotein may provide the antigenic stimulus. Similarly, it has been suggested that the presence of tissue precipitins in patients having ulcerative colitis may result from antigenic stimuli provided by the great number of microorganisms known to invade the tissue in this disease (15).

Whatever their origin, tissue antibodies have been found in normal animals and humans. Elevated levels of these antibodies have been found not only in human patients, but also in apparently normal healthy individuals. When these elevated levels exist, they are most apt to occur in individuals who give a biological false positive test for lues (cf. Tables I and VII) but bear no relationship, however, to the Wassermann antibody level. In the present studies, elevated levels of these tissue antibodies occurred in several apparently healthy individuals, which suggests that they may not be directly involved in the pathology of the so-called autoimmune diseases, or that additional conditions are essential. This point of view is corroborated by the findings of other investigators that indicate a poor correlation of the serological findings and tissue damage in experimental thyroiditis (13), experimental allergic encephalomyelitis (16) and experimental allergic testicular damage (17). Future work to determine the extent of correlation of tissue antibodies—circulating or possibly absorbed—with pathological changes in disease processes would be most useful. It is not unlikely that tissue damage may be produced either by antibodies which are absorbed by tissue cells and are, therefore, not present in the circulation, or by immunologically altered cells without the mediation of antibody. With respect to the first possibility it is known, for example, in certain particularly severe cases of erythroblastosis fetalis involving Rh antibody sensitization, that the circulating antibody levels in the mother rise markedly subsequent to delivery (18). The most plausible explanation for this finding is that the fetus may absorb antibody during gestation. The second possibility involving altered cells invokes an analogy with delayed hypersensitivity, but little evidence to support this thesis is currently available (19).

Tissue antibodies, at elevated levels, were invariably found in cases of systemic lupus erythematosus (SLE). Since the antigens used in the tests contained no detectable DNA, the question may be raised as to whether SLE sera react with cytoplasmic as well as with nuclear material. Other investigators (12, 20) have found that SLE sera react with isolated cytoplasmic constituents. It is well established, however, that cell nuclei contain appreciable quantities of non-nucleoprotein antigens soluble in 0.15 M sodium chloride (21) and highly reactive in complement-fixation tests with SLE sera (22). The antigens in this study contain such proteins and it is possible that the SLE and other sera reacted with both nuclear and cytoplasmic substances.

In contrast to the presence of tissue antibodies in patients with SLE, these antibodies were absent in cases of rheumatoid arthritis (Table VI). This result, together with concurrent findings of low levels of hemolytic complement in systemic lupus erythematosus patients and slightly elevated levels in rheumatoid arthritis patients (23) seems to cast doubt upon the assumption of common etiological mechanisms in these two clinical entities. If rheumatoid arthritis is associated with an autoimmune response, then the antibodies do not result in lowered complement levels.

Complement-fixing antibodies against γ-globulin in sera of patients with high titers of the rheumatoid factor have not been demonstrated in our laboratory. These findings do not support the premise that the rheumatoid factor is a typical antibody.

The tests with sera from patients with luetic infections have indicated a correlation of Wassermann antibody levels and tissue antibody levels. On the other hand, individuals who produce Wassermann antibody in the absence of luetic infections, so-called biological false positive reactors, often possess markedly elevated levels of tissue antibody (17 of the 71 had titers of 9 or greater against calf thymus, as indicated in Table VII), but these bore no relationship to the Wassermann antibody titer. The presence of Wassermann antibody in these reactors has been regarded as a sign of overt or latent immunological disease (24). Since systemic lupus erythematosus is the immunological disease par excellence and since practically all patients suffering from it have elevated levels of tissue homogenate antibody, but not necessarily of Wassermann antibody, it may well be that tissue homogenate antigen may provide serological evidence of greater validity for autoimmune disease processes than does Wassermann antigen.

With respect to the relation of infectious diseases and the development of tissue antibodies, humans with mumps or poliomyelitis, guinea pigs with Rocky Mountain spotted fever, and chimpanzees with typhoid fever all failed to show enhanced tissue antibody levels; a notable exception was a human with a fatal concurrent rickettsial and bacterial infection who developed high levels of tissue antibodies. A very recent study has indicated also that human patients with bacterial infection do not generally develop elevated levels of tissue antibodies (25). Rabbits inoculated intratesticularly with *T. pallidum* developed high Wassermann antibody titers but, unlike human patients with syphilis, developed only a very slight rise in titer against the antigens used in this study. This difference in serological response between humans and the rabbit may be related to the fact that experimental syphilis in the rabbit results chiefly in lesions of the skin, bones, and the eyes, but visceral lesions are virtually unknown (26). On the other hand, rabbits infected with *T. gambiense* or *T. rhodesiense* developed significant antibody increases against tissue homogenates but scarcely any rise in Wassermann antibody. It appears then that the autoantigens induced by trypanosomal infection in the rabbit are qualitatively different from those induced by treponemal infection.

In addition to the lack of enhanced tissue antibody levels in many patients with infectious diseases, the failure to find increased tissue antibody levels in experimental animals subjected to burns, X-irradiation, and large amounts of vaccines indicates that tissue damage per se does not lead to the appearance of tissue antibodies, but rather that the tissue changes leading to autoantigenicity are rather limited and possibly of a unique nature. The significance of the association, if any, in one patient between an extremely low level of complement and slightly elevated levels of tissue antibody levels is not readily apparent. In this individual as with other patients, the antibodies and complement measured in the circulation probably represent only one phase of a very complex and dynamic situation involving tissue alteration resulting in autoantigenicity, synthesis of antibodies, absorption of antibodies and complement fixation, and degradation or elimination of antibodies influenced by hormones and other factors.

SUMMARY

Normal sera of several animal species possess tissue antibodies demonstrable by complement fixation with calf thymus or rabbit liver homogenates. Enhanced levels of these antibodies have been found in patients with systemic lupus erythematosus and lues, and in animals experimentally infected with trypanosomes. They have not been observed in patients having rheumatoid arthritis or many infectious diseases, or in animals subjected to a wide variety of conditions. Coincidentally, the results have suggested that antibody levels against tissue homogenates may afford a more reliable index of overt or incipient autoimmune disease than does Wassermann antibody. Finally, the presence of these tissue antibodies in high titer in apparently normal healthy individuals lends additional credence to the concept that autoantibodies are not exclusively involved

in the pathological findings of the so-called autoimmune diseases.

ACKNOWLEDGMENT

Valuable contributions of sera of chimpanzees infected with *S. typhosa* were provided by Lt. Colonel Sidney Gaines, MSC; guinea pigs with experimental shigellosis by Dr. Samuel Formal; guinea pigs infected with the rickettsial agent of Rocky Mountain spotted fever by Miss Marilyn Bozeman; and burned rats by Dr. Ole Malm.

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THE EFFECT OF pH ON THE FILTRATION, REABSORPTION, AND EXCRETION OF PROTEIN BY THE RAT KIDNEY *

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In a paper discussing the protein content and acidity of human urine, von Hoesslin in 1909 suggested that a direct relationship existed between the hydrogen ion content and the protein content of human urine (1). Since then clinicians occasionally have commented on this relationship, some to confirm its existence (2, 3) and at least one to deny it (4). These comments usually have been based on the observation of patients thought to have proteinuria of the "benign" or "physiological" variety—e.g., orthostatic albuminuria. The present investigation was undertaken when a review of the pertinent publications since 1909 failed to reveal any formal laboratory study of the effect of pH on protein metabolism in the kidney. The study was designed to provide at least partial answers to two major questions. First, in mammals, do changes in the rate of urinary protein excretion accompany variations in the systemic and urinary hydrogen ion concentrations? Second, if such changes do occur, are they the result of changes in the filtration and/or reabsorption of protein within the kidney?

In these experiments the adult male rat was selected to represent the mammalian order, since rat urine is known to contain significant amounts of protein under normal conditions (5). In addition, the use of a laboratory animal afforded ready access to renal tissue in which the formation of "protein absorption droplets" (6) within the cells of the proximal convoluted tubules could be studied histologically.

The results of this investigation show that significant changes in urinary protein excretion accompany systemic acid-base disturbances in the male rat and suggest that this phenomenon is the result of changes in protein filtration at the glomerular level.

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MATERIALS AND METHODS

General experimental conditions. Male Wistar rats weighing 185 to 213 g were housed in individual metabolic cages. They were fed Purina Dog Chow pellets for at least 24 hours preceding each experimental period. During urine collection periods solid food was withdrawn to minimize contamination of the urine by food particles, and the animals were given a solution of 15 per cent glucose in distilled water, thus insuring adequate volumes (7) of relatively clean urine. The urines were collected from screened funnels beneath each cage over periods of time ranging from 8 to 24 hours. Toluene and mineral oil were not routinely used in the collection containers, since preliminary observations indicated that only minimal urinary pH changes took place in the absence of these preservatives. These urines provided a control baseline against which the urines from subsequent experiments were compared. Variations in the systemic and urinary pH's of these and additional animals were produced by the addition of 1 per cent NH_4Cl, 5 per cent $NaHCO_3$, 0.5 per cent $(NH_4)_2CO_3$, or 0.04 per cent acetazolamide (Diamox [1]) to the 15 per cent glucose in their drinking water. While the animals drank their assigned solutions, urines were again collected over a time interval equal in length to the previously established control period.

Analysis of urinary pH and protein excretion. Urinary volumes for each period were measured. Urinary and serum pH values were determined with a glass electrode pH meter within 1 hour after the collection terminated.

The Shevky-Stafford method (8) was chosen for routine use in determining the urinary protein concentration. This procedure consists of the addition of 2.5 ml of Tsuchyia's phosphotungstic acid reagent to a 4 ml aliquot of the supernatant from the centrifuged urine specimen to be analyzed. The resultant precipitate is allowed to flocculate undisturbed for 1 minute and is then centrifuged at a constant speed at room temperature for 5 minutes in a centrifuge tube designed for this procedure. At the end of this time, the volume of the protein precipitate is read directly from a scale etched

[1] Kindly provided by the American Cyanamid Company, New York, N. Y.

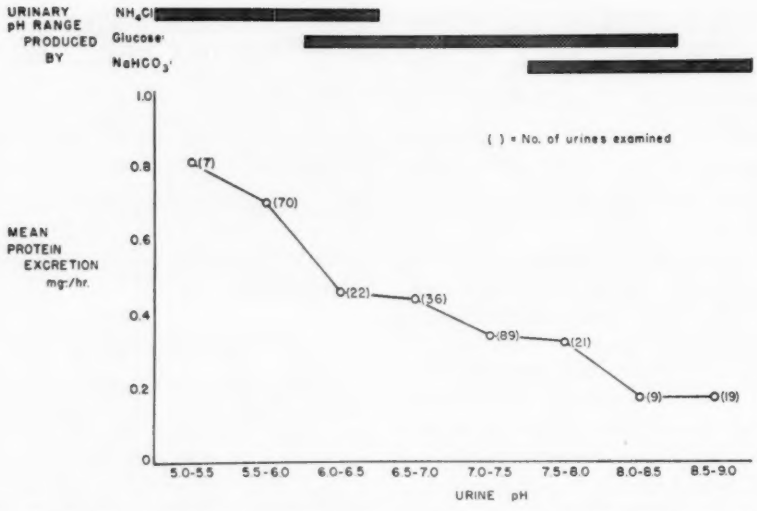

FIG. 1. THE MEAN RATES OF URINARY PROTEIN EXCRETION IN 273 URINES OF 100 RATS INGESTING 15 PER CENT GLUCOSE/WATER, PLAIN AND WITH 1 PER CENT NH_4Cl OR 5 PER CENT $NaHCO_3$ ADDED.

on each tube. This value is then used to determine the protein concentration of the specimen in milligrams per milliliter from a nomogram available for this purpose (9). The values obtained by this method agree closely with those obtained by the Kjeldahl procedure. Shevky and Stafford reported an average percentage error of 8.4 per cent in protein concentration of 30 specimens analyzed by both methods (8). A similar comparison among 10 specimens analyzed by both methods in our laboratory showed that the Shevky-Stafford method yielded results 4.4 ± 3 per cent higher than those obtained by the Kjeldahl procedure.

Random samples of individual rat serum and pooled urine specimens from animals partaking of each drinking solution were subjected to electrophoresis, using a barbital buffer at pH 8.6. Additional rats from each group were lightly anesthetized with ether and exsanguinated from the abdominal aorta in order to obtain blood for pH and serum for total CO_2 content.

Observation of the "protein absorption droplet." It is known that protein undergoing reabsorption in the proximal portion of the mammalian nephron can appear within the proximal convoluted tubule (PCT) cells in the form of droplets (6). Various methods have been employed for their demonstration, and of these the one using T-1824 [2] (Evans blue dye) as a serum protein label (10) was selected for this study. The protein absorption droplet, when formed from this dyed protein, can be seen microscopically within the PCT cells of unstained renal tissue.

[2] Kindly supplied as W 761-1 by the Warner-Chilcott Laboratories, N. Y.

Rats were injected intravenously with 10 mg of T-1824 and were then allowed to drink *ad libitum* one of the above-mentioned drinking solutions for a period of 24 ± 6 hours; during this time no solid food was furnished. After this the animals were sacrificed by exsanguination from the abdominal aorta, the kidneys removed, and frozen sections from these organs prepared and examined under the ordinary light microscope. In addition, the kidneys from a few rats which were not injected with the dye were prepared in a similar fashion and the sections examined either with the phase microscope or after Gram staining, these methods representing alternate ways by which the protein droplets can be demonstrated. Ten or more kidneys from rats drinking each of the various solutions were examined, and the size, number and distribution of the protein absorption droplets were observed.

RESULTS

Consistent and striking variations were observed in both urinary protein excretion rates and protein absorption droplet formation in these experiments. In general, protein excretion was higher in the more acid urines and lower in the more alkaline urines, as is shown in Figure 1. The data are given in Tables I and II; and the experimental results are presented in detail as follows.

Proteinuria and droplet formation in rats ingesting plain 15 per cent glucose and water. Un-

der normal conditions the male rat excretes about 0.7 ml per hour of urine containing 0.5 mg of protein (5). In this study, the mean hourly urinary volume was increased to 2.6 ml per hour by the addition of 15 per cent glucose to the drinking water (7). In the 76 urines from the 37 rats in this group, urinary protein excretion averaged 0.40 mg per hour, with a mean urinary pH of 7.23. In random samples of arterial blood the pH averaged 7.41 and the total serum CO_2 content, 22.6 mmoles per L. These results were taken as "baseline" values for comparison with the results obtained in the remaining groups of rats.

Figure 2A shows the usual degree of protein absorption droplet formation that was observed in the kidneys from rats of this glucose-and-water-fed group; the degree of this droplet formation served as a baseline for comparison with the remaining groups. Electrophoresis of the urinary proteins present in this urine revealed all serum protein fractions to be represented except for albumin.

Proteinuria and droplet formation during $(NH_4)_2CO_3$ ingestion. In 40 specimens from 26 rats in this group, the urinary volume averaged 3.1 ml per hour; urinary protein excretion, 0.36 ± 0.03 mg per hour; and urinary pH, 7.20. Only the volume was significantly increased when compared with the glucose group. Neither blood pH nor total serum CO_2 content was appreciably different. Droplet formation was identical with that in the control group.

Proteinuria and droplet formation during 1 per cent NH_4Cl ingestion. In 69 specimens from 58 rats receiving 1 per cent NH_4Cl in 15 per cent glucose and water, the urinary volume averaged 1.0 ml per hour; urinary protein excretion was increased to 0.75 ± 0.04 mg per hour. This rate was significantly different from that in the control group ($p < 0.005$). In the acid-fed rats the urinary pH was 5.68 (also significantly different), and determinations of pH and total serum CO_2 content on random blood samples averaged 7.28 and 14.9 mmoles per L, respectively. Accompanying this acidosis and increased proteinuria was a pronounced increase in the degree of PCT cell droplet formation (Figure 2B). Electrophoresis of urine from these rats disclosed small amounts of albumin and a 700 per cent increase in the α-globulin fraction over that noted in the glucose controls.

The reversibility of this NH_4Cl-induced proteinuria was demonstrated by alternating a group of four rats every 48 hours between the NH_4Cl and the $(NH_4)_2CO_3$ drinking solutions for a 20 day period. Proteinuria rose during NH_4Cl in-

TABLE I

Changes in urinary protein excretion rates in relation to urinary volume and pH

Regimen	No. of rats	No. of specimens	Mean urine vol.	Mean pH	Mean protein excretion rates
			ml/hr	*units*	*mg/hr*
15% Glucose/water (controls)	37	76	2.6	7.23	0.40±0.03*
0.5% $(NH_4)_2CO_3$	26	40	3.1	7.20	0.36±0.03
1% NH_4Cl	58	69	1.0	5.68	0.75±0.04
5% $NaHCO_3$	12	20	3.2	8.02	0.17±0.04
0.04% Acetazolamide	38	42	1.6	7.51	0.40±0.05

* SE = standard error of the mean.

TABLE II

Protein absorption droplet formation in relation to blood pH, urinary pH, and urinary protein excretion

Regimen	Mean total serum CO_2 content*	Blood pH	Urine pH	Urinary protein excretion	Protein droplet formation
	mmole/L				
15% Glucose/water (controls)	22.6 [3]	7.41 [4]	Normal	Normal	Normal
0.5% $(NH_4)_2CO_3$	24.5 [2]	7.38 [2]	Normal	Normal	Normal
1% NH_4Cl	14.9 [10]	7.28 [4]	Acid	Increased	Increased
5% $NaHCO_3$	33.3 [6]	7.58 [4]	Alkaline	Decreased	Decreased to absent
0.04% Acetazolamide	15.6 [14]	7.32 [4]	Slightly alkaline	Normal	Increased

* Numbers in brackets indicate the number of animals studied.

gestion and fell during $(NH_4)_2CO_3$ ingestion, as is shown in Figures 3 and 4. There was a high negative correlation of urinary pH and protein excretion in this experiment (R, − 0.83, p, 0.001).

Preliminary evidence suggesting that NH_4Cl may have the same effect on urinary protein excretion in man was found in the observation that two of four humans receiving NH_4Cl for other purposes (11) experienced a threefold increase in their urinary protein excretion.

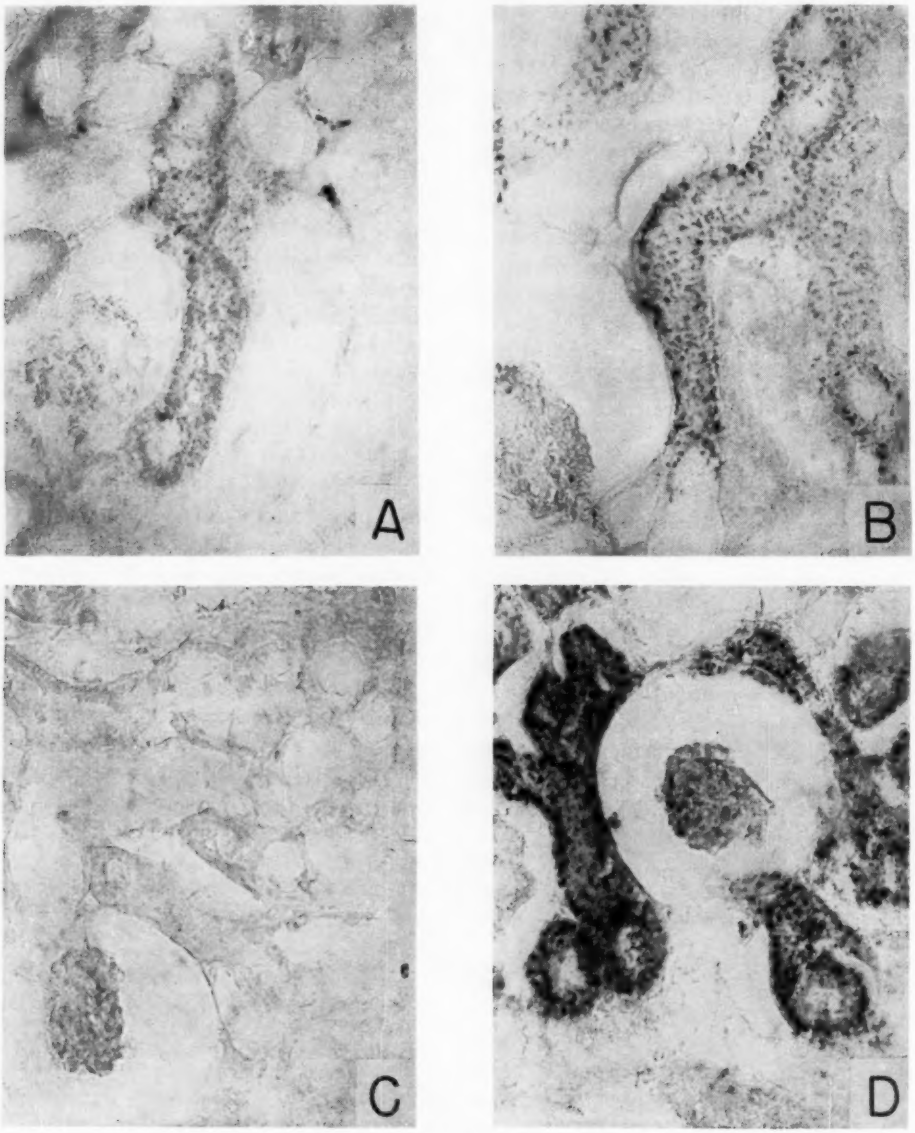

FIG. 2. PROTEIN ABSORPTION DROPLETS WITHIN THE PROXIMAL CONVOLUTED TUBULE CELLS OF RATS INJECTED 24 HOURS EARLIER WITH T-1824. A, after 24 hours on 15 per cent glucose and water (×385); B, after 24 hours on 1 per cent NH_4Cl (×385); C, after 24 hours on 5 per cent $NaHCO_3$ (×385); D, after 48 hours on 1 per cent NH_4Cl but 24 hours after the injection of dye.

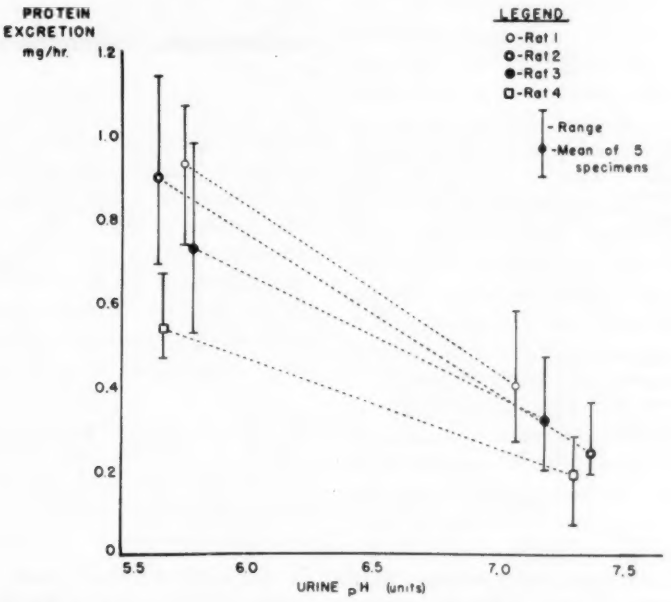

FIG. 3. MEAN URINARY PROTEIN EXCRETION RATES IN FOUR RATS ALTERNATED BETWEEN 1 PER CENT NH_4Cl AND 0.5 PER CENT $(NH_4)_2CO_3$ EVERY 48 HOURS FOR 20 DAYS. Acid urines were obtained during NH_4Cl ingestion only.

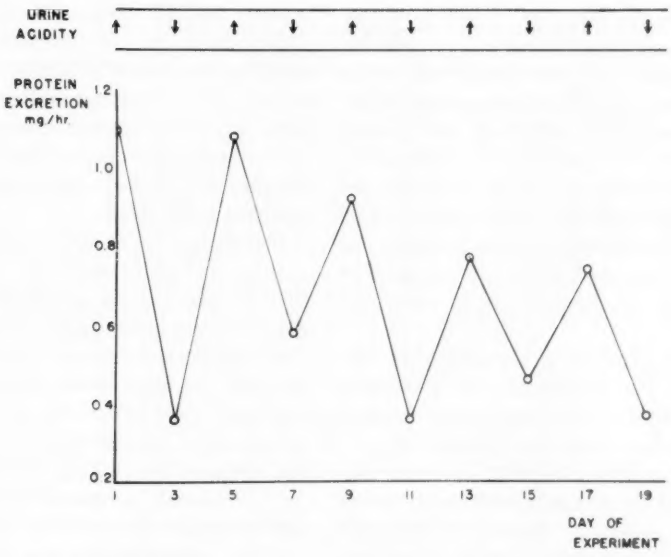

FIG. 4. ASSOCIATION OF INCREASED URINARY PROTEIN EXCRETION WITH INCREASED URINARY ACIDITY (LOWERED URINARY pH) IN RAT 1 OF FIGURE 3 OVER A 19 DAY PERIOD.

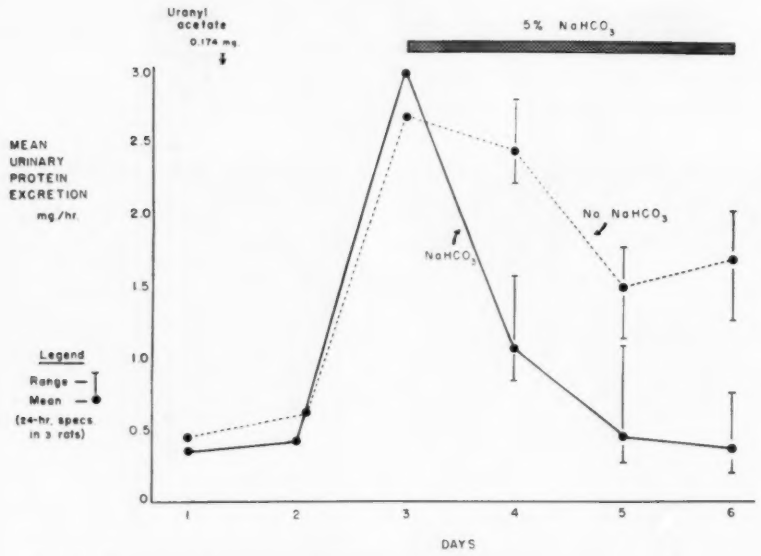

FIG. 5. EFFECT OF 5 PER CENT $NaHCO_3$ ON THE RATES OF URINARY PROTEIN EXCRETION IN THREE RATS WITH PROXIMAL CONVOLUTED TUBULE CELLS DAMAGED BY URANYL ACETATE, COMPARED WITH THREE SIMILAR RATS GIVEN ONLY 15 PER CENT GLUCOSE/WATER.

Proteinuria and droplet formation during $NaHCO_3$ ingestion. In 20 urine specimens from 12 rats ingesting the $NaHCO_3$ in 15 per cent glucose solution, hourly urine volumes averaged 3.2 ml. When compared with the glucose and water controls, the average hourly urinary protein excretion was decreased to 0.17 ± 0.04 mg, with a mean urinary pH of 8.02. The presence of a systemic alkalosis was confirmed by an average blood pH rise to 7.58 and a total serum CO_2 content mean of 33.3 mmoles per L. Electrophoresis of the urinary protein in the group again disclosed the presence of small amounts of all serum fractions except albumin.

The decreased proteinuria accompanying $NaHCO_3$ ingestion was associated with a virtually complete absence of protein absorption droplets within the kidneys of these rats (Figure 2C).

The observation that $NaHCO_3$ ingestion significantly lowered the urinary excretion of "physiological" protein raised the question: What might its effect be on "pathological" proteinuria? In an attempt to answer it, the following experiment was performed.

After the collection of control urines, six rats were given an intravenous injection of 0.175 mg of uranyl acetate, an amount known to damage severely the PCT cells of rats of similar size (12). Thirty-six hours later, 5 per cent $NaHCO_3$ was added to the glucose drinking solution of three of the six rats. A prompt and significant fall in the rates of urinary protein excretion was observed in these rats, while in the three animals continued on plain glucose and water, increased proteinuria persisted (Figure 5).

Proteinuria and droplet formation in 0.04 per cent acetazolamide ingestion. In 42 urines from 38 rats receiving the acetazolamide-glucose solution, the mean urine volume was 1.6 ml per hour. The mean urinary protein excretion did not differ from that observed in the plain glucose group, averaging 0.40 ± 0.05 mg per hour. However, the blood pH in these animals was reduced to 7.32 and the total serum CO_2 content to 15.6 mmoles per L. Urinary protein electrophoresis yielded a pattern similar to that found in the control urines.

When acetazolamide was alternated every 48 hours with the NH_4Cl solution, in one experiment, it neither produced nor maintained the increased proteinuria accompanying the ingestion of the

NH_4Cl solution (Figure 6). It did, however, produce and apparently maintain the acidemia.

DISCUSSION

The relationship between pH and proteinuria. The results of these experiments in rats support the clinical observations made in humans that changes in pH are accompanied by alterations in the rates of urinary protein excretion. In the acidemia and aciduria of NH_4Cl ingestion, urinary protein output was accelerated, and its prompt and repeated reversibility suggests that it was not the result of permanent damage to the kidney.

Consideration was given to other factors that might have influenced the excretion of urinary protein. Finlayson and Baumann (13) observed a direct correlation of urinary volume and protein content in male rats. In the present study, however, the highest rates of protein excretion occurred in association with the lowest average urinary volumes (the NH_4Cl-fed rats). Therefore, simple changes in volume did not seem to be responsible for the increased proteinuria. In the same report these authors also mentioned that ingestion of urea increased urinary protein excretion. In our experiments, however, it seemed not plausible to attribute the increased proteinuria accompanying NH_4Cl ingestion to an excess of ammonium ion, if not urea, since the daily intake of that ion in rats receiving NH_4Cl (with increased proteinuria) averaged 3.7 mEq per day, while its intake in rats receiving $(NH_4)_2CO_3$ (without increased proteinuria) averaged 5.2 mEq per day. Nor was the chloride ion apparently responsible, since proteinuria did not increase in rats receiving chloride ions in the form of KCl or NaCl in the experiments described by Masson, del Greco, Corcoran and Page (14).

If, then, hydrogen ion concentration is the key to this observed phenomenon, where and how might it exert its effect? An answer was sought in the changes found in the formation of the protein absorption droplets in the various experimental groups.

The protein absorption droplet. Oliver and others have intensively studied the droplets which form within cells of the PCT during protein reabsorption. Working with Straus, he extracted the droplets from the PCT cells of rats injected with egg albumin and found that the amount of the protein within the droplets was higher than that in the remaining cellular substrate (15). From

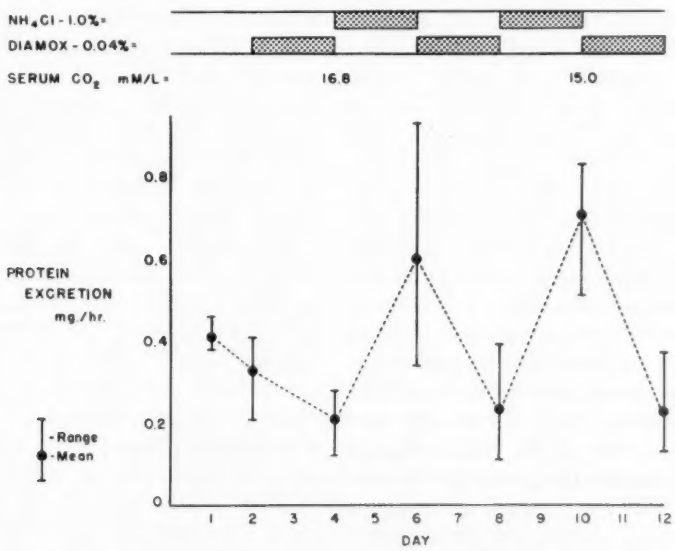

FIG. 6. FAILURE OF ACETAZOLAMIDE INGESTION TO PRODUCE OR MAINTAIN INCREASED URINARY PROTEIN EXCRETION PRODUCED BY NH_4Cl INGESTION IN A GROUP OF THREE RATS.

this and other studies Oliver has concluded that the protein absorption droplet represents an intracellular accumulation of protein, appearing whenever the normal mechanisms of cellular protein disposal are inadequate (6). This inadequacy may be the result of either an increased demand imposed on the cells by the reabsorption of increased amounts of protein or a deficiency in the ability of the cells to dispose of normal amounts of reabsorbed protein (6). From this it follows that a simple assessment of the size and number of these droplets in a given kidney does not permit any conclusion regarding the rate or the amount of protein undergoing reabsorption, unless further information is made available about the conditions under which the droplets were formed. In the present study, this information was sought in parallel experiments as the need arose and will be presented in abbreviated form when the significance of droplet formation in each of the experimental groups of rats is discussed below.

Suitability of T-1824 as a protein-absorption droplet indicator. The fact that T-1824 can be found in droplet form within the PCT cells of animals which have been injected with the dye does not necessarily mean that protein reabsorbed from the glomerular filtrate is also present within the droplet, although the available evidence strongly suggests that this is the case. *In vitro,* T-1824 has been shown to bind rapidly (within 50 seconds) to serum protein, primarily albumin (16), with a bond that is approximately three times as strong as the peptide linkages of the protein molecule itself (17). This union cannot be disrupted by incubating dye-labeled protein with liver slices (18). *In vivo,* the injection of T-1824 in doses up to 2.5 times greater than the amount used in these present studies does not result in the appearance of free dye in the serum (19) or in the urine (20); nor can dye be detected within the PCT cells immediately after its injection (19).

To test the possibility that T-1824 might leave more readily the protein of the tubular fluid to enter absorption droplets that were already present, 10 mg of T-1824 was injected intravenously into a series of rats 24 hours after the intraperitoneal injection of egg albumin. More than 7 and less than 24 hours elapsed before any droplets containing dye could be found in the kidneys of this group of serially-sacrificed rats, and even after 24 hours colored droplets were only sparsely scattered through their PCT cells. However, when these kidneys were examined with the Gram stain technique, a myriad of protein droplets was seen. These results imply that T-1824 is not freed from filtered protein at the cells' tubular border to enter the cell in the unbound state.

Interest in the pH changes occurring in these animals led to a study of the effect of pH on the ability of protein to bind T-1824 by means of *in vitro* use of bovine albumin solutions adjusted to pH's of 6.7 and 7.9. Eight moles of dye was added to each mole of protein present, a dye: protein ratio which closely approaches the saturation level for protein binding of dye at pH 7.4 (21). Less than a 10 per cent difference was found, with slightly more dye being bound at the lower pH, a difference considered insufficient to produce the degree of variation observed in droplet colorations in these present experiments. Therefore, from the evidence available, T-1824 appeared to be a suitable indicator for the detection of protein absorption droplets in this study.

Droplet formation and its significance in the NH_4Cl experiments. The addition of 1 per cent NH_4Cl to a 15 per cent glucose drinking solution was found to increase the formation of protein absorption droplets within the rat kidney. Referring to Oliver's hypothesis mentioned above, this increase could signify either an increase in the amount of protein undergoing reabsorption or a decrease in the ability of the cells to dispose of the reabsorbed protein. An attempt was made to ascertain the ability of the PCT cells in rats to dispose of reabsorbed protein.

Examination, under the phase microscope, of renal sections from undyed rats showed that absorption droplets were increased after 24 hours of NH_4Cl ingestion. If, at this point, rats were injected with T-1824 and maintained for a second 24-hour period on NH_4Cl, their kidneys were found to contain numerous, intensely colored protein droplets (Figure 2D). Under the phase microscope no droplets of undyed protein could be detected in other areas of the cytoplasm of these cells. The fact that droplet coloration in this experiment was greater than that observed in the kidneys from dyed rats after only 24 hours on NH_4Cl made it difficult to assume that the dyed

protein had entered and mixed with the undyed protein present within each droplet. It appeared that the undyed protein had been eliminated from the PCT cells to make way for the accumulation of the dyed protein, implying that these cells were able to continue protein disposal while under the influence of NH_4Cl.

Thus it seemed likely that the increased droplet formation within the kidneys of rats ingesting NH_4Cl was the result of increased amounts of protein passing through the cells, and in this instance indicated increased protein reabsorption. Coupling this information with the observation that these animals were also excreting increased amounts of protein in their urines, the inference may be made that increased amounts of protein were being filtered at the glomerular membrane.

Droplet formation and its significance in the $NaHCO_3$ experiments. Protein absorption droplets were very scarce or were completely absent in the kidneys from rats that had ingested $NaHCO_3$, implying either decreased reabsorption of protein or accelerated disposal of protein by the cells. To distinguish between these two possibilities, the PCT cells of an additional group of rats were damaged by the injection of uranyl acetate. Sodium bicarbonate was still effective in reducing urinary protein excretion under these circumstances despite the cellular damage and the disruption of protein reabsorption mechanisms, as have been described in uranium nephritis by Goodman and Baxter (12). Since the action of $NaHCO_3$ apparently is not dependent on the integrity of the PCT cells, it seems more likely that it reduced proteinuria in these experiments by reducing the amount of protein passing through the glomerular membrane. It was concluded that the decreased proteinuria of rats ingesting $NaHCO_3$ was the result of diminished glomerular protein filtration. In the light of this conclusion, it is of interest to recall the clinical observations of Schultz and Collier (22), Fox and McCune (23), and others who have described remissions in the nephrotic syndromes of their patients following massive alkali loading.

Droplet formation and its significance during acetazolamide ingestion. Droplet formation in the PCT cells of rats ingesting acetazolamide was identical in all respects with that observed in the kidneys from animals ingesting NH_4Cl. Systemic acidosis was also present in both groups of animals. Yet the urinary excretion of protein was unchanged by acetazolamide ingestion. When acetazolamide instead of NH_4Cl was furnished to rats for 24 hours before and for 24 hours after the injection of T-1824, no droplets of undyed protein were found, and droplet coloration was intensified. This again suggested that there was no severe impairment in the ability of the cells to dispose of protein during acetazolamide ingestion. Thus, increased protein filtration was implicated. Since increased urinary protein excretion was not observed in these animals, the reabsorption of protein during acetazolamide ingestion may have been sufficient to compensate for the suspected increase in protein filtration. It is equally possible, on the other hand, that some other effect of acetazolamide counteracted the effect of its acidemia on the increased filtration of protein to maintain protein excretion at normal levels.

Since the primary purpose of this present study was to determine the effect on proteinuria of a simple acid or alkali load and since spontaneous impairment of carbonic anhydrase activity has not been described in mammals, further exploration of the effect of acetazolamide on renal protein metabolism was not performed at this time.

Localization of the hydrogen ion effect. The task of demonstrating an effect of hydrogen ion concentration on either the filtration or the reabsorption of protein within the rat kidney would have been considerably easier were it possible to measure the rates of these processes directly. In the absence of a more adequate method, it was necessary to approach the problem indirectly, using the degree of protein droplet formation in the variety of experimental circumstances described above. In both the NH_4Cl and $NaHCO_3$ groups of rats, the results of this approach suggested, more strongly than not, that the observed change in urinary protein excretion was primarily the result of a concomitant change in the rate of protein filtration at the glomerulus. The experiments did not delineate whether this is a direct effect of hydrogen ion on the glomerular membrane or an indirect effect on renal blood flow, on the physical or electrostatic configuration of the serum protein molecules, or on some other mechanism involved in protein filtration.

SUMMARY

Changes in blood acidity and urinary pH were produced in rats by the ingestion of 1 per cent NH_4Cl, 5 per cent $NaHCO_3$, and 0.04 per cent acetazolamide (Diamox) in a 15 per cent glucose-and-water drinking solution. The concomitant rate of urinary protein excretion was measured. During NH_4Cl and $NaHCO_3$ ingestion a negative correlation was found between urinary and serum pH and the degree of proteinuria. In general, the more acid the urine and the blood, the greater the proteinuria. The increased proteinuria observed during NH_4Cl ingestion was promptly reversible when a neutral or alkaline drinking solution was substituted, and the urinary and systemic pH consequently was increased.

A study of protein absorption droplets within the proximal convoluted tubule cells of kidneys from each group of rats was made. An increase in droplet formation was observed in the presence of a lowered serum pH. The observation that T-1824-labeled protein replaced undyed protein in these droplets during established acidosis suggested continuing protein reabsorption during NH_4Cl ingestion. This in turn suggested increased protein filtration as the primary cause for the increased proteinuria in these animals. A decrease in droplet formation was observed in the presence of an elevated serum pH. The ability of $NaHCO_3$ to reduce the proteinuria of uranium nephritis in rats suggested that tubular cell integrity was not essential for its action and that the decrease in proteinuria observed during alkalosis was not primarily the result of increased reabsorption but of decreased glomerular filtration.

These experiments demonstrate that the physiological proteinuria of the male rat varies with changes in the animal's acid-base equilibrium and suggest that this phenomenon is related primarily to concomitant changes in the filtration rather than to reabsorption of protein within its kidneys.

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LIPOPROTEIN PATTERN AS A FUNCTION OF TOTAL TRIGLYCERIDE CONCENTRATION OF SERUM *

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The occasional occurrence of lactescent or milky serum in patients with certain diseases caused interest and speculation at least as long ago as 1799 when Mariet described turbid sera in some patients with diabetic acidosis (1). The light-scattering fat particles or chylomicrons causing lactescence are present in modest numbers after a fat meal but become scarce in normal postabsorptive sera. In certain pathological states, however, chylomicrons occur in excessive numbers, giving rise to a characteristic milky appearance of the sera.

In a previous study, lactescence was reported to be directly proportional to the total triglyceride concentration in serum (2). Turbidity was invariably present when total triglyceride fatty acid concentration exceeded 20 mEq per L. As triglyceride concentration increased further, a progressively greater proportion of cholesterol and phospholipids also occurred in the particulate lipid fraction which could be readily removed by flotation of unaltered serum at moderate speeds in the ultracentrifuge.

Serum lipids are now known to exist in a spectrum of lipoproteins of varying density, from very low density particulate chylomicrons composed chiefly of triglycerides, through various classes of low density lipoproteins relatively rich in cholesterol, to high density lipoproteins in which phospholipids are prominent (3).

Interest in various classes of low density lipoproteins has been stimulated because of their possible role in the etiology of coronary arterial disease (4). Studies from this laboratory (5–7) have shown serum triglyceride concentration to be intimately associated with this disease. Antonis and Bersohn have also found elevation of serum triglycerides in ischemic heart disease (8). From the known composition of very low density lipoproteins, it might be inferred that their presence in abnormally high concentrations would be associated with increased concentration of total serum triglycerides.

The present study was undertaken to learn whether total triglyceride concentration might determine not only the fraction of lipids present in chylomicrons but also the partition of lipids among the various low density lipoproteins. At the same time the lipoprotein composition of the relatively clear subnatant fluid after removal of the chylomicron "cream" layer of lactescent sera could be determined. This clear fraction was previously analyzed *in toto* (2), but no analysis of lipoproteins was made.

METHODS

Serum was obtained from medical school personnel without known disease and from patients in the wards and clinics of the Grace-New Haven Community Hospital. Markedly lactescent sera were obtained from patients with essential hyperlipemia, tuberous xanthomata, or diabetic acidosis. Slightly or moderately lipemic sera were found in patients who had suffered a myocardial infarction but who had none of the stigmata of hyperlipemia. One coronary patient had normal lipids. Patients with hypothyroidism or with essential hypercholesterolemic xanthomatosis provided the sera with high cholesterol but normal triglycerides. The subjects and patients, all of whom were studied in the postabsorptive state, are listed in Table I, together with the serum lipid data and a brief clinical identification.

Blood was drawn from an antecubital vein and the cells separated from the serum within 2 hours of the venipuncture. The serum was subjected to 4 successive centrifugations, in the no. 40 rotor of a Spinco model L preparative ultracentrifuge, with removal of various fractions in the following manner.

1. Unaltered serum was centrifuged at 18,000 rpm (20,000 G) for 1 hour in order to remove most of the

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particulate lipids, chiefly chylomicrons. The bottom one-half to two-thirds of the clear subnatant fluid was withdrawn through a needle into a syringe; an aliquot was taken for analysis. The chylomicron composition was calculated by subtracting the concentration of lipid components in this layer from their concentration in the original serum. The assumption was made that no significant migration of lipoprotein other than chylomicrons occurred as a result of such mild ultracentrifugation (2). In very lipemic sera, a slight opalescence remained in the subnatant fraction indicating that not all visible lipid particles had been removed. Therefore, for want of a better term, the lipids removed by this first centrifugation, previously referred to as "insoluble lipids" or "emulsified lipids" (2), will here be called "light" chylomicrons.

2. The subnatant serum (usually 10 ml) obtained by Procedure 1 was, without further alteration, centrifuged at 40,000 rpm (105,000 G) for 22 hours. A clear separation between the supernatant and subnatant lipids was marked by a water-clear middle zone. A Spinco tube slicer was used to slice the tubes through the clear zone at the junction of the upper and middle thirds. The entire supernatant fraction was carefully transferred to a 5 ml volumetric flask with several washings of physiological saline, made to volume, and analyzed for lipids. This layer was markedly lipemic when the original serum was lipemic. The lipids so isolated are designated "heavy" chylomicrons, although it is recognized that they represent a transitional stage between chylomicrons and denser lipoproteins. Assuming the nonprotein solvent density of serum to be 1.006 (9), the "light" chylomicrons plus the "heavy" chylomicrons together constitute the fraction of lipids of density less than 1.0006 and are designated as $D < 1.006$.

3. The subnatant solution from Procedure 2 was transferred to a 10 ml volumetric flask, with several washings of physiological saline. This was adjusted to D 1.019 according to the method of Havel, Eder and Bragdon (9), and spun at 40,000 rpm for 22 hours in the ultracentrifuge (105,000 G). The top third of the fluid was removed as in step 2 and analyzed. This fraction contained lipoproteins D 1.006–1.019.

4. The subnatant fluid was removed as in step 3, adjusted to density 1.063 by the method of Havel and coworkers, and centrifuged at 40,000 rpm for 22 hours. The top third of the fluid, constituting the lipoproteins of D 1.019–1.063, was removed for analysis. The subnatant fluid was also analyzed and designated as fraction $D > 1.063$. During all centrifugations the centrifuge chamber was refrigerated in order to maintain the temperature of the sera below 18° C.

The original serum and each of the fractions were extracted with 2:1 (vol/vol) chloroform-methanol. Water was added to create a two-phase system and the chloroform phase was analyzed for total fatty acids (10), for total cholesterol by the method of Abell, Levy, Brodie and Kendall (11), and for lipid phosphorus by a modification of the method of Fiske and Subbarow (12). The triglycerides were calculated as the difference between total fatty acids and fatty acids of cholesterol esters and phospholipids, using assumptions previously described (10). Triglycerides were expressed as milliequivalents per liter of triglyceride fatty acid. Since free cholesterol was not determined, it was assumed that 72 per cent of the cholesterol was esterified. The known differences in the ratio of free to total cholesterol in different lipoproteins (3) would introduce only a small error in the estimation of triglycerides. The triglycerides so calculated include the free fatty acids, which may account for appreciable amounts of the triglycerides in the fraction of density > 1.063. The concentration of components of each density fraction was expressed in terms of its concentration in whole undiluted serum.

RESULTS

The concentration of triglyceride fatty acids, cholesterol and the cholesterol-phospholipid ratios of each serum are shown in Table I. The sera are listed in order of increasing triglyceride concentration. A triglyceride concentration greater than 5.5 mEq per L is considered the upper limit of normal by the standards of this laboratory (6, 7, 13). A brief clinical description of each subject is given. Except for E. E., who was studied twice, each serum was obtained from a different person.

The lipid concentration of the uncentrifuged sera varied greatly. The triglyceride fatty acid concentration varied from 2.1 to 200 mEq per L, and the cholesterol from 179 to 792 per 100 ml. The cholesterol of whole serum tended to increase as the triglyceride increased, but with a great deal of variation. In some instances cholesterol was markedly elevated in the presence of normal triglycerides and in others was normal in the presence of somewhat increased concentrations of triglycerides. The cholesterol to phospholipid ratio varied from 0.71 to 1.33. As previously noted (2), slight to moderate lactescence appeared as the triglyceride fatty acid concentration approached 20 mEq per L, and became marked when triglycerides exceeded this concentration.

The total concentration of lipids recovered in the lipoprotein fractions varied from 79 to 95 per cent of their concentration in the original serum, the incomplete recovery resulting most likely from the summation of small losses frequently encountered through leakage around the caps of the lustroid tubes in which the fractions were centrifuged. The losses affected total fatty acid, cholesterol and

TABLE I

Lipids of whole serum and of fractions of various density, arranged in order of increasing triglyceride concentration of whole serum *

Subject	Age Sex	Original serum			Density fraction <1.006						Density fraction 1.006–1.019			Density fraction 1.019–1.063			Density fraction >1.063			Comment
					"Light" chylomicrons			"Heavy" chylomicrons												
		TG	TC	CP	TG	TC	CP	TG	TC	CP	TG	TC	CP	TG	TC	CP	TG	TC	CP	
R. D.	30 F	2.1	459	1.31	0.7	0	†	0.1	3.6	1.44	0	10.2	2.04	0.8	300	1.66	0.5	58	0.68	Tendinous xanthoma
T. F.	29 M	2.2	222	0.94	0	5	0.2	0.7	13	1.04	0.2	16	1.60	0.7	105	1.55	1.2	57	0.61	Normal
P. R.	30 F	2.3	179	0.88	0	1	0.4	0.4	9	0.90	0.1	10	1.35	0.7	77	1.40	1.6	52	0.64	Normal
M. G.	60 F	2.6	229	0.72	0	0	†	0.9	15	1.53	0.3	16	1.10	0.6	88	1.62	1.1	58	0.38	Thrombophlebitis
D. R.	26 M	3.0	246	1.03	0	2	0.1	1.0	12	0.96	0.3	16	2.10	0.8	130	1.67	0.7	46	0.63	Normal
J. C.	57 M	3.9	218	1.23	0	0	†	1.1	15	1.36	0.3	11	1.14	0.6	119	1.43	0.8	42	0.53	Coronary heart disease
J. L.	25 F	4.7	197	0.83	0.6	0	†	4.2	12	1.20	0.2	19	1.26	0.4	63	1.40	0.7	26	0.52	Normal
A. W.‡	50 F	5.4	672	1.20	1.6	0	†	0.7	20	1.33	0.4	56	1.60	1.9	490	1.64	1.5	62	0.65	Hypothyroid
F. E.	35 M	5.5	232	1.07	2.8	0	†	0.9	10	0.35	0.1	14	1.87	0.7	133	1.90	1.0	56	0.57	Normal
W. H.	66 M	5.6	203	0.99	0.2	0	†	1.5	25	1.61	0.2	9	0.94	1.1	100	1.67	1.7	22	0.35	Coronary heart disease
J. P.	55 M	8.8	362	1.12	0	8	0.36	4.0	42	0.96	0.4	34	1.44	0.8	181	1.66	0.7	36	0.48	Coronary heart disease
H. T.	52 M	9.4	243	1.08	0.6	8	†	2.8	31	0.90	0.1	9	0.85	1.3	124	1.77	1.4	20	0.43	Coronary heart disease
A. R.	73 F	9.7	248	0.77	1.3	12	0.23	3.1	32	0.86	0.8	30	1.20	0.6	51	1.14	1.2	40	0.38	Coronary heart disease
R. L.	44 M	16.9	386	1.24	3.5	23	†	6.9	103	0.98	1.6	97	1.39	0.9	68	1.30	1.2	30	0.46	Coronary heart disease
B. B.‡	35 M	21.7	470	1.10	8.1	56	0.64	6.1	162	1.58	1.7	66	5.3	1.0	37		2.2	31		Tuberous xanthoma
O. G.	41 M	31.3	384	0.92	8.2	149	13	12.4	112	0.83										Coronary heart disease
E. M.	43 M	35.4	792	1.28	8.6	204	2.06	16.4	308	1.23	1.7	62	1.24	2.3	120	1.14	1.2	22	0.34	Essential hyperlipemia
F. T.	40 M	38.3	318	0.71	12.4	63	0.38	16.4	112	0.75	0.4	10	1.00	1.7	66	1.90	2.6	44	0.49	Essential hyperlipemia
B. C.	67 F	46.0	450	0.87	15.9	124	0.87	22.2	217	1.10	0.3	10	1.33	1.0	24	1.20	2.4	32	0.34	Essential hyperlipemia
M. A.	45 F	49.1	549	0.78	13.9	109	1.09	23.9	246	0.95	1.3	22	1.12	0.8	17	0.93	3.15	61	0.37	Essential hyperlipemia
E. E.	38 M	53.7	561	1.09	36.7	303	1.36										2.8	31	0.44	Essential hyperlipemia
J. A.	60 F	67.0	529	0.94	51.0	352	1.07	10.7	104	0.82	†	†	†	1.6	25	1.06	2.9	22	0.68	Essential hyperlipemia
E. E.	38 M	79.4	601	1.33	71.1	490	1.66	4.0	31	0.46	0.6	14.3	4.4							Essential hyperlipemia
A. R.	58 F	95.0	761	0.89	50.2	315	0.80	30.2	260	0.94	0.3	9	0.9	1.4	26	1.30	3.4	38	0.58	Diabetes; cor. heart dis.
O. P.	60 M	129.4	642		105.8	467											2.6	23	0.31	Diabetic acidosis
H. H.	36 M	200.6	572	0.73	184.4	458	0.80							0.8	33	1.65	3.2	40	0.59	Diabetic acidosis

* TG = triglyceride fatty acids in milliequivalents per liter; TC = total cholesterol in milligrams per 100 ml; CP = cholesterol to phospholipid ratio. Blank spaces signify that a particular fraction was not measured.

† No recovery of cholesterol or phospholipid or both.

‡ Age is approximate.

phospholipids about equally. Triglyceride recoveries were more variable, reflecting the greater error in their determination. In spite of the rather numerous opportunities for losses, a clear relationship between triglyceride concentration and lipoprotein pattern can be detected.

With increasing triglyceride concentration of whole serum, there were small increases in the triglycerides of the fractions D > 1.006, but the total triglyceride found in these three fractions together—D 1.006–1.019, 1.019–1.063, and > 1.063—never exceeded 6 mEq per L. When total triglyceride concentration exceeded 6 mEq per L the triglycerides in the "heavy" chylomicrons increased, but never above 30 mEq per L. The greatest triglyceride concentrations of very lipemic sera were found in the "light" chylomicrons, which contained as much as 184 mEq per L, 90 per cent of the triglyceride in the most lactescent serum.

The cholesterol concentration in the most dense fraction, D > 1.063, was fairly uniform throughout the entire group of sera, varying only between 20 and 62 mg per 100 ml. There was an inconstant tendency for the cholesterol in this fraction to be lower in the lactescent than in the clear sera.

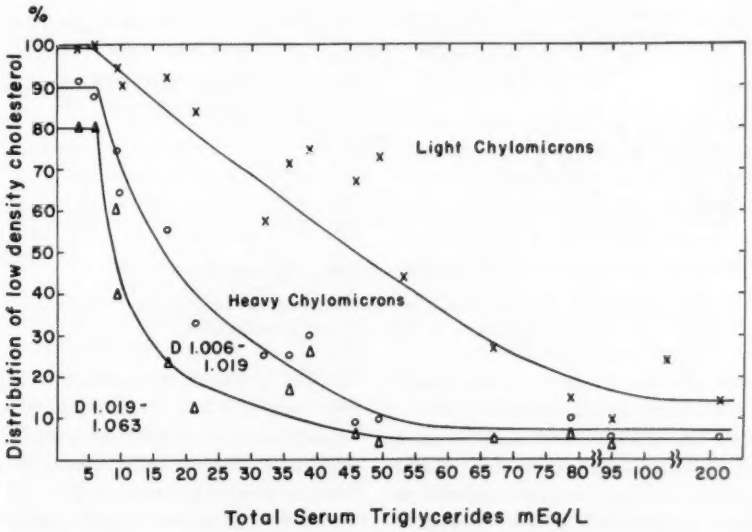

FIG. 1. EFFECT OF SERUM TRIGLYCERIDE CONCENTRATION, EXPRESSED AS TRIGLYCERIDE FATTY ACIDS, UPON THE DISTRIBUTION OF CHOLESTEROL AMONG LOW DENSITY LIPOPROTEIN FRACTIONS. The vertical axis represents the total cholesterol of D < 1.063 in per cent. For each serum the distribution of cholesterol within the various low density subfractions is indicated by three symbols arranged vertically above the triglyceride concentration of that serum: triangles, D > 1.019; circles, D > 1.006; X's, all the low density fractions except the easily removed "light" chylomicrons. The 100 per cent line represents all cholesterol of D < 1.063.

The first set of points (triglyceride concentration of 3.3 mEq per L) represents the average of the first eight sera from Table I. The second set of points (triglyceride concentration of 5.6 mEq per L) represents the average of the next two sera. The third set of points (triglyceride concentration of 9.3) represents the average of three patients whose triglyceride concentrations ranged from 8.8 to 9.7 mEq per L. All other points represent individual sera. The lines were drawn to give the best visual fit to the various points. The mid-portion of line separating the "light" from the "heavy" chylomicrons was drawn to conform to the lower rather than the upper X's in recognition of the intermixture of these two layers during manipulation of the centrifuge tubes as a technical source of error, particularly at triglyceride concentrations of about 40 mEq per L. The vertical distances between the lines represent the relative cholesterol concentrations in any one category as indicated.

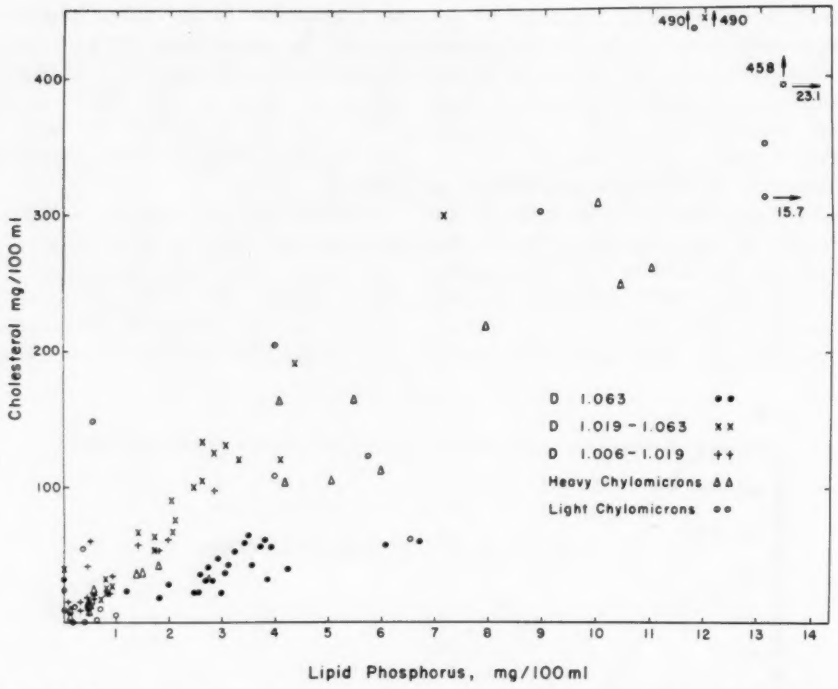

FIG. 2. CHOLESTEROL TO LIPID PHOSPHORUS RELATIONSHIP IN VARIOUS LIPOPROTEIN FRACTIONS IN A VARIETY OF NORMAL AND LIPEMIC SERA.

A remarkable influence of triglyceride concentration on the distribution of cholesterol among the various fractions of D < 1.063 is apparent in Table I. The cholesterol, which in the presence of normal triglycerides was largely concentrated in the fraction D 1.019–1.063, appeared, with increasing triglyceride concentration, in fractions of successively lower density. This relationship was even more prominent when the distribution of cholesterol among the low density fractions (D < 1.063) was expressed as percentage of the total recovered low density cholesterol. Figure 1 shows the progressive shift of cholesterol from higher to intermediate density categories and, finally, out of the latter into the chylomicrons. In massively lactescent sera, 80 per cent or more of the cholesterol in all of the fractions of D < 1.063 was present in the "light" chylomicrons, leaving the intermediate density fractions relatively devoid of lipid.

The cholesterol to phospholipid ratio, calculated by using a factor of 25 for converting the lipid phosphorus into phospholipid, was characteristically low in the fraction of D > 1.063, ranging from 0.31 to 0.68, tending to be a little lower in lactescent than in clear sera. The ratio was higher in the four lower density fractions, in most instances varying between 1.0 and 2.0. The low ratios found in the "light" chylomicrons of sera with normal total triglycerides cannot have much significance because of the great error of this ratio attendant upon the low concentrations of both cholesterol and phospholipid in this fraction. The cholesterol to phospholipid ratios in the various fractions are shown graphically in Figure 2. A rather constant linear relationship of cholesterol to lipid phosphorus is seen in the D > 1.063 fraction, and a different but also rather constant relationship in the fractions of D 1.019–1.063 and 1.006–1.019. Between these two extremes are found most of the points representing the more variable cholesterol to phospholipid relationships of the fractions D < 1.006, the "heavy" and "light" chylomicrons.

Each serum was centrifuged in a single tube; the reproducibility of the methods of separation

cannot therefore be determined from comparison of duplicate centrifugations but can be estimated by comparing results of different sera of similar triglyceride concentrations in this and a previous study (2). The "light" chylomicron fraction is uniformly of almost negligible lipid concentration in clear serum with normal triglyceride concentration, but is somewhat more variable in lactescent sera, as is evident from the scatter of the X's about the line of best fit in Figure 1. In all fractions only 7 of 89 points, either shown individually in Figure 1 or used in obtaining the averaged points, departed from the appropriate line by more than 10 per cent on the vertical axis.

DISCUSSION

The present study shows the expected close relationship between total serum triglyceride concentration and lipoprotein pattern and permits the prediction of the latter from the concentration of total cholesterol and total triglyceride. The lipids were fractionated into those of high density, $D > 1.063$, and low density, $D < 1.063$. The latter were further divided into four subfractions. Although the concentration of low density cholesterol was related to the total serum cholesterol concentration, the relative distribution of low density cholesterol among the various subfractions appeared to be directly dependent upon serum triglyceride concentration. In the presence of normal triglycerides, the low density cholesterol and phospholipids were largely in the fraction D 1.019–1.063. With increased concentrations of total triglyceride, fatty acids above 6.0 mEq per L cholesterol and phospholipids were found in fractions of progressively lower density to an extent determined by the total triglyceride concentration. All increments of triglyceride above 6.0 mEq per L occurred in the light-scattering fraction of $D < 1.006$.

Table II shows the percentage distribution of low density cholesterol among the four fractions of $D < 1.063$ at any given concentration of total triglyceride. The figures in this table were derived from the curves in Figure 1, which in turn were drawn as the lines visually best representing the individual points. By assuming a concentration of 40 mg per 100 ml for the cholesterol present in the fraction of $D > 1.063$, a fraction which seems to be little if any influenced by triglyceride concentration, and by subtracting this figure from the total cholesterol, the concentration of cholesterol in the combined fractions of $D < 1.063$ can be estimated to within 20 mg per 100 ml. If the triglyceride concentration is known, the absolute concentration of cholesterol in each of the four density fractions, which together comprise the group $D < 1.063$, can then be estimated from Table II.

TABLE II

Distribution of cholesterol among low density lipoprotein fractions as a function of serum triglyceride concentration

Triglyceride fatty acids	Low density cholesterol			
	<1.006*		1.006–1.019†	1.019–1.063‡
	"Light"	"Heavy"		
mEq/L	%	%	%	%
5	1	9	10	80
10	6	24	28	42
20	20	38	22	20
30	32	40	14	14
40	43	38	10	9
50	54	36	5	5
60	65	27	3	5
70	75	18	2	5
80	80	13	2	5
100†	86	7	2	5

* Corresponds to S_f20–40,000 (3).
† Corresponds to S_f12–20.
‡ Corresponds to S_f0–12.

The relationship between triglyceride concentration and lipoprotein pattern suggests a strong affinity between triglyceride-rich chylomicrons and lipoproteins of D 1.019–1.063. The inference from these data that cholesterol and phospholipids were removed from the D 1.019–1.063 fraction to lower density fractions is at least plausible. The exact nature of this affinity is not known, although the data from this and a previous paper (2) suggest a solubility distribution of cholesterol between an aqueous lipoprotein phase and a lipid chylomicron phase. Fredrickson and Gordon (14) have reviewed in detail evidence for affinity between both α- and β-lipoproteins and chylomicrons and the significance of this affinity in the clearing of chylomicrons from the circulation. The slight diminution of high density cholesterol in lipemic sera and the somewhat greater concentration of phospholipids relative to cholesterol in chylomicrons, as compared with lipoproteins of D 1.019–1.063, are consistent with the possible attraction of α- as well as β-lipoproteins to chylomi-

crons. A fraction of serum cholesterol that is easily dissolved by organic solvents (15, 16) has been described—a phenomenon which might be used in support of the concept of a fraction of serum cholesterol easily dissolved in chylomicrons. The emulsifying action of phospholipids on fats is frequently mentioned, but the possible role of cholesterol in maintaining a fine state of emulsification of oils (17) is usually neglected. A chief function of lipoproteins may well be that of maintaining insoluble triglycerides in a state of fine emulsification and in a physical state promoting their interaction with a biochemical system that functions in aqueous solutions. The fine dispersion of the chylomicrons of even the most lipemic serum is impressive and difficult to create in artificial emulsions.

All of the lipemic sera of the present study must be considered abnormal in that the hypertriglyceridemia persisted for at least 14 hours after the last meal, usually sufficient time for the alimentary lipemia of the previous day to have subsided (18). The changes described here must be distinguished from the changes accompanying normal alimentary lipemia characterized by a small rise in triglyceride concentration restricted to the lipoproteins of $D < 1.019$ (19, 20), although an increase in the chylomicron cholesterol and an associated decrease in the cholesterol of the remaining serum may occur during the intravenous infusion of fat emulsions (21) in association with a much greater increase in triglyceride concentration than usually occurs after a fat meal.

The association between triglyceride concentration and lipoprotein pattern may have important implications in the field of coronary arterial disease. The presence of abnormally great concentrations of low density lipoproteins in sera of patients with coronary arterial disease, described by Gofman and colleagues (4), has been amply confirmed, but the separation between the normal and coronary populations on the basis of lipoproteins of the classes S_f12–20 and 20–100 has not proved to be very striking (22). A better separation was found by Gofman on the basis of the atherogenic index, an index based on a mathematical analysis of lipoproteins in which lipoproteins of the class S_f100–400 are given relatively more weight than those of greater density (22). Since triglycerides constitute an increasingly important part of lipoproteins of decreasing density, total triglyceride concentration provides an indication of lipoprotein concentration already weighted in favor of those of very low density, including those of S_f400 and greater. Nichols, Lindgren and Gofman (23) have described a similar relationship between total lipid and atherogenic index. Total lipid, however, is composed largely of cholesterol and phospholipid in pure hypercholesterolemia, and largely of triglyceride in lipemic sera. There was little relationship between total cholesterol and its distribution among low density lipoproteins in the present study. Since total lipid is a rough indicator of triglyceride concentration, particularly in lipemic sera, Nichols and colleagues were probably in effect measuring the same relationship as that described here between triglyceride concentration and lipoprotein pattern.

Recent studies from this laboratory demonstrated that serum triglyceride fatty acid concentrations above 5.45 mEq per L were characteristic of 80 per cent of a group of patients with coronary arterial disease (6, 7). This concentration was exceeded by only 5 per cent of young men of 20 to 29 years and by about 35 per cent of middle-aged men without known coronary arterial disease. Another communication contains a detailed comparison of the separation between a normal and coronary population, achieved on the basis of triglyceride concentration, with that effected on the basis of various categories of lipoproteins reported by others (7). It is perhaps more than coincidence that the concentration of triglyceride which best separates the normal from the coronary population coincides with the total maximal capacity of lipoproteins of $D > 1.006$ for triglycerides, and with the concentration of triglyceride at which the shift of cholesterol from higher to lower density lipoproteins begins to occur.

Lipoprotein patterns have also been reported to be characteristic of disease states. Gofman, Rubin, McGinley and Jones (24) noted that essential hyperlipemia and tuberous xanthoma were characterized by very low concentrations of lipoproteins S_f0–12, corresponding to the density class 1.019–1.063, but by great elevations of the classes S_f20–100 and 100–400 of density < 1.006. In tendinous xanthomatosis there was an increase chiefly in the S_f0–12 class. Nephrosis was asso-

ciated with an increase in all lipoprotein densities up to S_f100–400. These authors suggested that lipoprotein structure be considered the standard diagnostic feature of these diseases.

The relationships reported in the present paper are in accord with the findings of Gofman and associates. Patients with essential hyperlipemia as a rule have serum triglyceride concentrations great enough to be associated with a marked decrease of cholesterol in the fraction D 1.019–1.063 and an increase in the very low density cholesterol. On the other hand, the high cholesterol in the presence of normal triglycerides, characteristic of sera from patients with tendinous xanthomatosis, would be expected, on the basis of the present paper, to be concentrated in the fraction D 1.019–1.063. Although no cases of nephrosis were included in the present study, Baxter, Goodman and Havel (25) reported that the lipoprotein pattern of nephrotic sera could be predicted from the triglyceride and cholesterol concentrations in a manner entirely consistent with the findings reported here.

On the basis of the present study it seems possible that the relative prominence of various categories of lipoproteins depends solely upon the total serum triglyceride concentration and thus upon the metabolism of triglyceride. The findings are in accord with the hypothesis that a delay in the normal rate of removal of triglycerides from the circulation, with a consequent rise in plasma triglyceride concentration, is responsible for the accumulation of very low density lipoproteins in plasma in certain diseases.

SUMMARY

Twenty-six clear and lactescent sera from normal persons and a variety of patients with disorders of lipid metabolism were analyzed for cholesterol, triglycerides, and cholesterol to phospholipid ratio of whole serum and of various lipoprotein fractions. Lipoproteins were ultracentrifugally separated into fractions of density (D)> 1.063, D 1.019–1.063, D 1.006–1.019 and D< 1.006. The latter were further divided into "heavy" and "light" chylomicrons.

Triglyceride concentration of whole serum had a marked effect upon lipoprotein pattern. All triglyceride fatty acid increments over 6 mEq per L occurred in the fraction D < 1.006. As triglycerides increased above this concentration, the relative concentration of cholesterol among the various fractions of D < 1.063 shifted out of the fraction D 1.019–1.063 and into fractions of progressively lower density. On the basis of data presented, it was possible to predict the cholesterol concentration of the various lipoprotein fractions from the concentration of triglycerides and cholesterol of whole serum.

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STUDIES ON ALBUMIN SYNTHESIS: THE EFFECTS OF DEXTRAN AND CORTISONE ON ALBUMIN METABOLISM IN RABBITS STUDIED WITH ALBUMIN-I^{131} *

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Following plasmapheresis, the depressed serum albumin concentration rapidly returns to normal (1) indicating alterations in albumin synthesis or degradation. Both dextran administration (2–6) and elevation of serum globulin levels produced by means of hyperimmunization (7, 8) are associated with hypoalbuminemia; a colloid osmotic regulatory mechanism has been suggested as responsible for the reciprocal changes in serum proteins observed after hyperimmunization (7, 9). If such a mechanism does exert control over the concentration of serum albumin, alterations of albumin metabolism either in the rate of synthesis or in the rate of degradation might be expected when osmotically active molecules other than albumin are added to the circulation. The present study represents an attempt to define, more specifically than heretofore reported, the changes in albumin metabolism resulting from such a procedure. Dextran was administered to rabbits to depress albumin concentration. After the development of hypoalbuminemia, the rates of albumin synthesis and degradation were studied and compared with control values. Cortisone acetate was then given to increase the rate of albumin degradation (10), and the rates of albumin synthesis were remeasured.

METHODS

Female rabbits were used in all studies. Animals were kept in metabolism cages and fed a standard rabbit diet. Lugol's solution was added to the drinking water daily. The distribution and metabolism of albumin were studied by means of I^{131}-labeled rabbit serum albumin. Assays of I^{131} in plasma, stool and urine were made for a control period of 10 to 14 days following intravenous administration of albumin-I^{131}. Following the control period, 1.0 to 1.5 g of dextran (Laros, lot H., average molecular weight 188,000) dissolved in 20 ml 0.9 per cent saline was injected i.v., once daily in 10 animals. After 15 to 30 days, the animals received a second injection of albumin-I^{131} while the daily dextran infusions were continued, and observations were continued for another 12 to 18 days. Six of the 10 animals were then started on cortisone acetate (3 mg per kg per day, s.c.) and dextran infusions were continued for another 9 to 14 days. At the end of this period a third injection of albumin-I^{131} was made to remeasure albumin synthesis, degradation and pool size.[1] In two other rabbits, cortisone and dextran were administered simultaneously from the beginning of the study for a period of 15 days. Two other animals were infused with 20 ml of 0.9 per cent saline for 15 and 24 days. Two of the animals receiving dextran alone were studied again with albumin-I^{131} 6 and 8 weeks after the termination of the original studies to determine whether the prolonged dextran infusions had had any lasting effects on albumin synthesis. During periods of cortisone administration rabbits received 0.25 g streptomycin and 75,000 U procaine penicillin intramuscularly every other day and 5 to 8 mEq potassium chloride in the water daily, of which the animals consumed 2 to 3 mEq per day. Injections of albumin-I^{131} were made into an ear vein. Five- to eight-tenths ml heparinized venous blood was obtained from the opposite ear 6 and 10 minutes after injection, at daily intervals for 1 week, and every second or third day thereafter. Blood samples taken for assay were obtained just prior to the daily injections of dextran.

Rabbit albumin was separated from whole serum as previously described (10) and occasional lots of rabbit albumin preparation, no. 7305,[2] were employed after re-

* This material was presented in part at the meeting of the Eastern Section of the American Federation for Clinical Research on December 10, 1959. This investigation was supported in part by Grant no. A-2489 from the United States Public Health Service.

[1] Reinjections of albumin-I^{131} were made at such time intervals and dose levels that the residual activity from previous injections was negligible and did not affect the subsequent calculations.

[2] Purchased from Pentex Inc., Kankakee, Illinois.

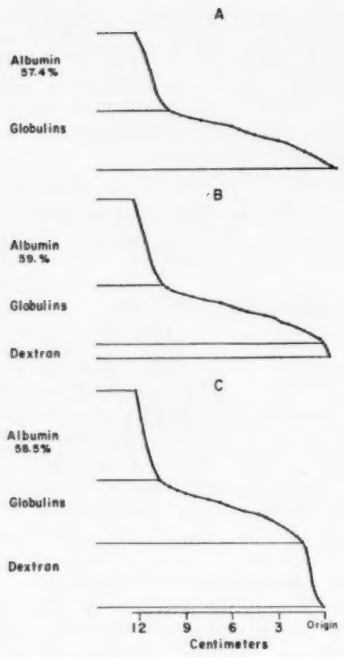

FIG. 1. CONCENTRATION CROSSPLOTS OF THE INTERFEROMETRIC PATTERNS OF RABBIT SERUM (3.50 G/100 ML) INCUBATED WITH THE DEXTRAN CONCENTRATION VARYING FROM 0 (A), 1.20 G/100 ML (B), AND 2.25 G/100 ML (C). SEE TEXT.

purification. The I^{131}-labeling procedure has been described previously (11). All lots of albumin-I^{131} were tested in normal control animals to insure against the use of a lot that contained any significant amounts of denatured labeled protein (10).

Dextran solutions were prepared by dissolving 120 g in 1,500 ml of normal saline and filtered through a bacterial filter. Plasma dextran concentrations were determined by the method of Roe (12). Urine was tested for protein by precipitation with cold 20 per cent trichloroacetic acid.

Plasma, urine and stool samples were assayed for radioactivity in a well-type scintillation counter with a sensitivity of 9.7×10^5 cpm per μc I^{131} above a background of 170 cpm. Total plasma protein was determined by a micro-Kjeldahl method. Concentration of plasma albumin was determined by boundary electrophoresis with a Kern microelectrophoresis apparatus. In this method, 0.2 ml serum or plasma was diluted to 0.8 ml with Michaelis-veronal buffer at pH 8.6 and the solution was dialyzed against the buffer for 30 minutes prior to electrophoresis for 40 minutes at 80 v. The presence of dextran did not interfere with the determination, since the albumin fraction remained constant at dextran concentrations ranging from 0.1 to 2.0 g per 100 ml. Dextran itself resulted in a clearly differentiated interference pattern at the initial boundary (Figure 1).

Plasma volume was determined from the space of distribution of albumin-I^{131} at 6 and 10 minutes after injection. Total exchangeable albumin and the extravascular: intravascular distribution of albumin were determined as previously described (11). The rate of albumin metabolism during the control period was determined from

TABLE 1

Albumin distribution *

Animal	Weight			Hematocrit			Plasma volume			TEAS			Albumin partition		
	N	D	D+C	N	D	D+C	N	D	D+C	N	D	D+C	N	D	D+C
	kg			*%*			*ml/kg*			*mgl/kg*			*% intravascular*		
4	4.08	4.08		36.8	28.5		27.9	34.8		74.5	105.8		37.4	33.0	
6	3.63	3.86		38.6	25.8		37.8	47.2		94.5	126.5		40.1	37.4	
9	5.45	5.45		33.6	24.5		34.5	45.0		86.6	112.5		39.8	40.1	
16	6.46	6.37		39.0	32.7		29.1	35.2		70.4	90.0		41.4	39.2	
22	5.05	5.91	5.23	37.8	27.0	29.2	30.9	33.8	57.9	77.8	109.0	131.0	39.7	31.0	44.4
7	4.08	4.66	4.32	37.8	23.8	18.3	38.0	41.4	82.5	100.5	119.0	171.5	37.9	34.8	48.2
10	4.78	4.54	4.20	35.6	23.2	18.4	29.7	40.4	66.6	77.8	126.6	174.5	38.2	31.8	38.3
11	5.23	5.00	4.78	42.3	29.6	22.0	24.6	35.2		69.1	108.2	127.5	35.6	32.5	
18†	3.87	4.54	4.10	43.0	33.0	25.8	33.6	36.8	58.5	93.4	98.0	132.0	36.6	37.6	44.4
46	4.54	4.54	4.66	32.0	25.2	18.5	35.5	42.8	59.6	100.0	122.0	165.0	36.2	35.1	36.2
21	5.94		5.54	39.8		26.4	28.3		53.0	76.4		114.2	37.3		46.4
24	5.24		5.13	36.3		23.6	33.2		60.5	86.5		133.8	38.4		45.2
Mean value	4.86	4.90	4.75	37.7	27.3	22.8	31.9	39.3	62.7	84.0	111.8	143.7	38.2	35.3	43.3
±SE				0.9	1.1	1.5	1.2	1.6	3.7	3.1	3.8	8.8	0.5	1.0	1.7
p Value					<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.02	<0.001
Per cent change (N−D)/N					−27.6			+23.2			+33.1			−7.6	
Per cent change D−(D+C)/D						−16.5			+59.5			+28.5			+22.7

* TEAS = total exchangeable albumin space. N = control study. D = dextran study. D+C = dextran and cortisone study. SE = standard error of the mean. All p values are based on comparisons between the control (N) and experiment values (D and D+C).

† Rabbit 18 was studied while it received saline injections between the control and dextran study. The experimental periods, however, are compared with the initial control values.

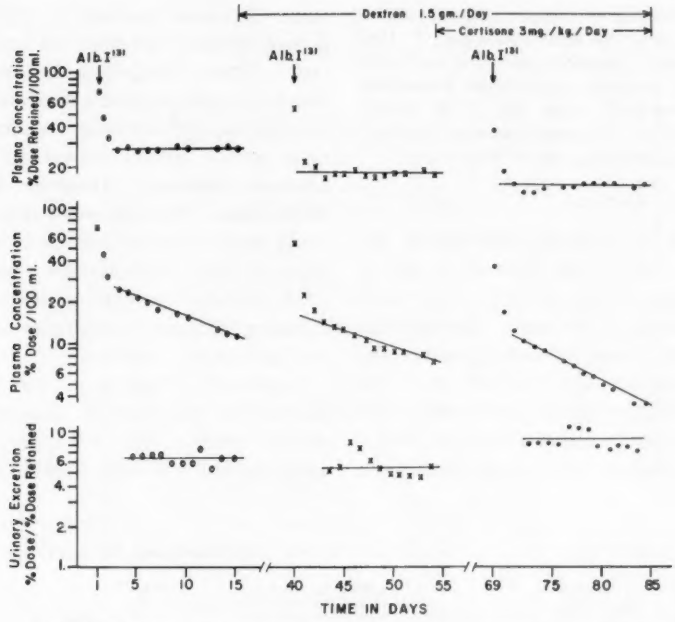

FIG. 2. TYPICAL SET OF CURVES (RABBIT 10) SHOWING DISTRIBUTION, PLASMA DECAY, AND URINARY EXCRETION CURVES DURING THE CONTROL PERIOD AND FOLLOWING THE ADMINISTRATION OF DEXTRAN AND DEXTRAN AND CORTISONE.

the product of the clearance of plasma-I^{131} and the plasma albumin concentration. This technique has been described in detail in previous reports (10, 13, 14). It has been demonstrated that the clearance method can be applied in nonsteady state conditions (10, 13, 15). The total amount of albumin degraded over each period was obtained from the sum of the daily values of the amount of albumin degraded, as determined by the clearance method. During the control periods, the plasma albumin concentrations remained constant, and it may be assumed that steady state conditions, with respect to albumin metabolism, were satisfied, and that the quantity of albumin synthesized was equal to the quantity degraded. In previous studies the amount of albumin synthesized during an experimental period was calculated from the difference between the total amount degraded and the change in the total exchangeable albumin pool during the period. In this study the rabbits were "equilibrated" with the test substances before reinjection, and the experimental data for albumin degradation were derived from subsequent injections of albumin-I^{131}. Since the plasma albumin values changed only slightly following the reinjections, albumin synthesis was assumed to closely parallel albumin degradation. These observations do not permit an exact evaluation of degradation or synthesis during the period of equilibration prior to reinjection of albumin-I^{131}. However, the control and experimental values for albumin degradation and the changes in pool size are known and indicate the net albumin bal-

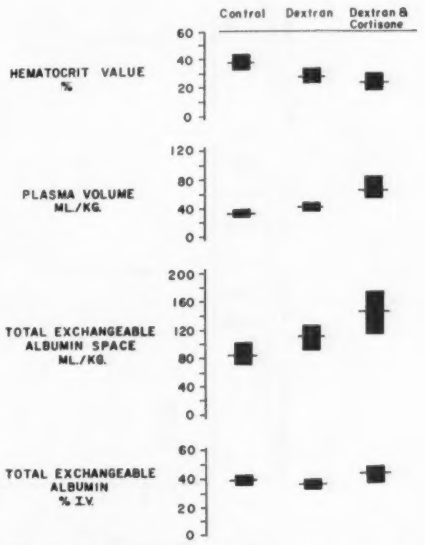

FIG. 3. ALBUMIN DISTRIBUTION. The mean values (horizontal lines) and ranges (solid bars) for hematocrit value, plasma volume, total exchangeable albumin space and per cent of total exchangeable albumin located intravascularly during control, dextran and dextran and cortisone periods. See text and Table I.

ance as well as the direction of change in albumin degradation and synthesis during the equilibration period. Previous reports have dealt adequately with the use of albumin-I^{131} as a tracer for endogenous albumin metabolism in both steady and nonsteady states (10, 11, 13, 15–19).

The significance of the differences between the mean values was determined according to the t test (20).

RESULTS

Data pertaining to albumin distribution are summarized in Table I and Figures 2 and 3. Weights of the animals showed no specific trend throughout any portion of the study. During dextran administration, mean hematocrit values declined in 12 animals by 27.6 per cent while the plasma volume increased by 23.2 per cent. Following institution of cortisone treatment in the dextran-treated animals, the mean hematocrit value decreased further by 16.5 per cent and plasma volume increased an additional 59.5 per cent. These changes in hematocrit values and plasma volume suggest a decrease in red cell mass or shrinkage of erythrocytes after dextran treatment and an increase in red cell production with cortisone treatment. However, alterations in the intravascular distribution of red cells and plasma could explain these findings as well. The mean apparent total exchangeable albumin "space" increased by 33.1 per cent on dextran treatment and a further 28.5 per cent increase was observed upon the addition of cortisone.

Significant changes in the intravascular-extravascular partition of albumin occurred. In control animals, 38.2 ± 0.5 per cent of the total albumin pool was located within the plasma space.

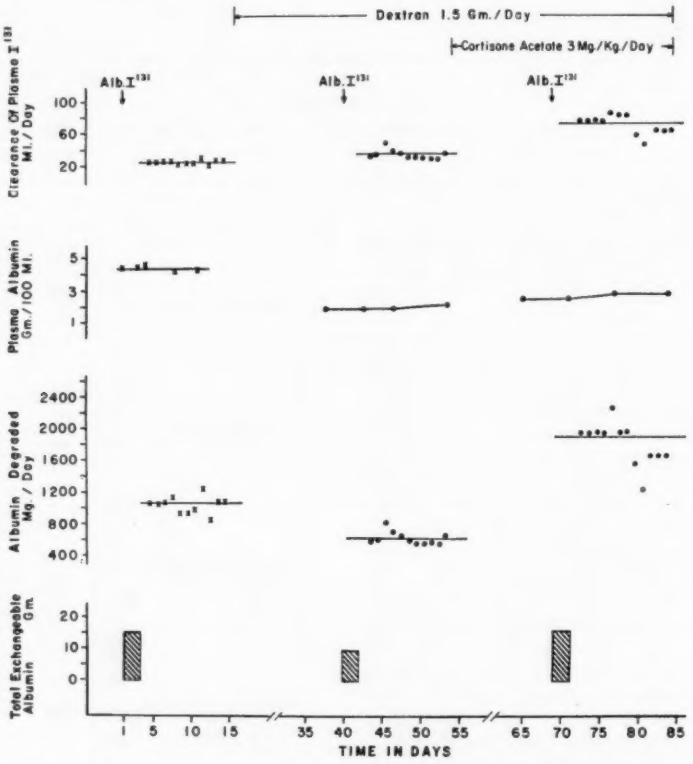

FIG. 4. TYPICAL ANIMAL EXPERIMENT (RABBIT 10). Following dextran administration, a slight rise in the renal clearance of plasma-I^{131} occurred; plasma albumin concentration, albumin degradation and the exchangeable albumin pool decreased. Upon the addition of cortisone, the clearance of plasma-I^{131} rose considerably, plasma albumin concentration rose slightly, and there was an increase in albumin degradation and the exchangeable albumin pool.

TABLE II

Albumin metabolism *

Animal	Total protein			Albumin			TEA			Albumin degradation			Days of study			Clearance		
	N	D	D+C	N	D	D+C	N	D	D+C	N	D	D+C	D	D+C	TD	N	D	D+C
		g/100 ml			*g/100 ml*			*g/kg*			*mg/kg/day*						*ml/day*	
4	6.38	4.48		3.93	2.55		2.92	2.70		245	216		17			25.4	29.8	
6	6.97	4.80		4.27	2.55		4.05	3.22		258	189		17			21.9	26.9	
9	6.01	4.55		3.37	2.55		2.92	2.86		181	156		30			29.3	28.2	
16	7.00	5.12		4.08	3.00		2.88	2.70		265	236		16			41.7	48.4	
22	6.98	4.52	5.30	4.28	2.45	3.25	3.33	2.67	4.26	293	185	440	17	17	43	34.4	43.1	71.1
7	6.69	4.38	4.51	3.86	2.40	3.00	3.87	2.85	5.14	263	171	469	24	14	53	27.8	33.8	60.5
10	6.66	3.40	4.00	4.33	1.85	2.40	3.39	2.33	4.20	224	134	450	25	14	54	24.7	32.5	67.8
11	6.22	4.11	4.50	3.66	2.20	2.80	2.52	2.38	3.57	235	166	274	24	10	50	33.3	38.9	47.6
18†	7.18	5.05	4.71	4.28	3.05	3.00	4.03	2.95	3.95	333	256	391	12	9	34	30.1	35.4	55.7
46	7.15	4.90	4.02	4.16	3.00	2.50	4.16	3.66	4.06	288	242	304	24	12	44	29.4	38.4	61.0
21	6.92		4.84	3.88		2.65	2.96		3.04	217		379		15		33.4		73.9
24	6.70		4.21	3.72		2.39	3.23		3.16	208		283		15		29.9		62.7
Mean value	6.74	4.53	4.51	3.99	2.56	2.75	3.36	2.83	3.93	251	195	374				30.1	35.5	62.5
± SE	0.11	0.17	0.16	0.28	0.12	0.11	0.16	0.09	0.24	12	13	27				4.9	6.8	3.0
p Value		<0.001	<0.001		<0.001	<0.02		<0.02	=0.01		<0.01	<0.001					=0.05	<0.001
Per cent change (N − D)/N		−32.8			−35.8			−15.5			−22.3						+17.9	
Per cent change D−(D+C)/D			−0.4			+7.4			+38.4			+91.8						+76.1

* TD = total duration of dextran administration. TEA = total exchangeable albumin. N = control study. D = dextran study. D + C = dextran and cortisone study. SE = standard error of the mean. All p values are based on comparisons between the control (N) and experimental values (D and D + C).

† Rabbit 18 was studied while it was on saline injections between the control and dextran study. The experimental periods, however, are compared with the initial control values.

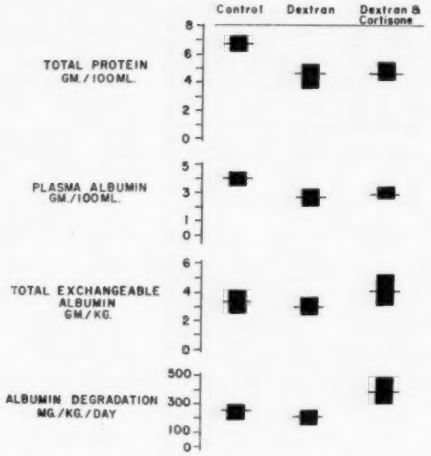

FIG. 5. ALBUMIN METABOLISM. The mean values (horizontal lines) and ranges (solid bars) for total protein, albumin concentration, total exchangeable albumin and albumin degradation during control, dextran, and dextran and cortisone periods. See text and Table II.

Following the administration of dextran, this fraction decreased slightly to 35.3 ± 1.0 per cent, and in animals receiving cortisone and dextran the intravascular compartment increased to 43.3 ± 1.7 per cent of the total exchangeable albumin.

The data for albumin metabolism are shown in Table II and Figures 4 and 5. After dextran administration, the mean total plasma protein fell 32.8 per cent, with essentially no change after cortisone therapy. Dextran administration resulted in an average decrease in albumin concentration of 35.8 per cent, while plasma globulin fell by only 28.4 per cent. This finding does not confirm previous reports which indicated a greater fall in the globulin than in the albumin fraction (2, 3, 21). After cortisone administration, the mean albumin concentration actually rose by 7.4 per cent. The mean total exchangeable albumin pool declined 15.5 per cent after dextran administration and rose 38.4 per cent upon the addition of cortisone.

A decrease in albumin degradation of 22.3 per cent was observed following the administration of dextran. Upon the addition of cortisone, albumin degradation increased by a mean of 49.0 per cent over control values and 91.8 per cent over prior dextran values.[3] Dextran infusions resulted not

[3] The effects of combined dextran and cortisone acetate administration were calculated after the rabbits received cortisone acetate for 9 to 17 days, and the data were derived from the third injection of albumin-I^{131}. In Rabbits 10, 18 and 46, daily observations on the excretion of I^{131} derived from the second dose of tracer albumin were continued after the start of cortisone acetate administration. In these 3 rabbits the excretion of I^{131}, corrected for the fraction of the second dose remaining within the animal, rose rapidly and after 4 to 8 days

TABLE III

Albumin distribution and metabolism *

Animal	Weight		Hematocrit		Plasma volume		TEAS		Albumin partition	
	N	E	N	E	N	E	N	E	N	E
	kg		*%*		*ml/kg*		*ml/kg*		*% intravascular*	
4†	4.08	4.54	36.8	39.3	27.9	26.0	74.5	73.0	37.4	35.5
6†	3.63	4.08	38.6	37.5	37.8	34.9	94.5	97.0	40.1	35.5
18‡	3.87	3.87	43.0	40.0	33.6	34.6	93.4	96.0	36.6	35.9
60‡	5.91	6.14	37.8	38.8	33.5	33.9	88.6	85.6	37.6	39.4

	Total protein		Albumin		TEA		Albumin degradation		Days of study	Clearance	
	N	E	N	E	N	E	N	E		N	E
	g/100 ml		*g/100 ml*		*g/kg*		*mg/kg/day*			*ml/day*	
4†	6.38	6.47	3.93	4.24	2.92	3.10	245	219		25.4	23.5
6†	6.97	6.78	4.27	4.04	4.05	3.90	258	229		21.9	23.2
18‡	7.18	6.93	4.28	4.10	4.03	3.90	334	333	15	30.1	29.5
60‡	5.82	5.84	4.00	4.04	3.60	3.50	280	262	24	41.3	40.2

* N = control. E = experimental. TEAS = total exchangeable albumin space. TEA = total exchangeable albumin.

† Received no treatment during experimental periods which followed by 6 weeks (Rabbit 4) and 8 weeks (Rabbit 6) the discontinuation of dextran treatment in these animals.

‡ Received only daily saline injections during experimental period.

only in a decrease in the amount of albumin degraded but also in a mean decrease of total exchangeable albumin of 530 mg per kg. Thus, while degradation decreased, a diminution in synthesis must have taken place to an even greater extent to result in this loss of total exchangeable albumin. Upon the administration of cortisone, changes in the opposite direction occurred—namely, an increase in degradation and an increase in the exchangeable albumin pool by a mean of 1,100 mg per kg. Thus, during the 9 to 17 days of combined dextran-cortisone treatment, albumin synthesis exceeded degradation to a considerable degree. The clearance of plasma-I^{131}, resulting from albumin degradation,[4] averaged 30.1 ± 4.9 ml per day during the control period. An increase to 35.5 ± 6.8 ml per day occurred following dextran administration, and a further more marked increase to 62.5 ± 3.0 ml per day was obtained following the administration of cortisone with dextran.

Concentrations of circulating dextran ranged between 1.2 and 2.1 g per cent during dextran administration and were not significantly altered in the presence of the expanded plasma volume following cortisone therapy.

Table III indicates the results in two animals given saline infusions and in two animals restudied 6 and 8 weeks, respectively, after discontinuing dextran. Neither group showed any marked changes from the control periods.

Most of the animals in the initial study were followed long enough before reinjection so that an accurate estimate of total excretion of radioactivity could be obtained. Between 93 and 106 per cent of the injected dose was accounted for in urine, stool, withdrawn blood, and that still present in the original total albumin space of distribution. No evidence for a very slowly exchanging albumin compartment, as has been suggested by others (19), was obtained from these studies.

DISCUSSION

While plasma volume expansion clearly follows the infusion of dextran (2–4), little is known concerning the effects of dextran infusions upon the distribution and metabolism of plasma proteins. The plasma protein pool has been reported to decrease (2, 3, 22), to remain essentially unchanged (23), or to increase (24), and while there is disagreement as to whether albumin or globulin is more affected by dextran infusion, the plasma concentrations of both have been noted to decrease (2–6, 21–25).

In the present studies, dextran produced the expected plasma volume expansion and also an expansion of the apparent volume for albumin distribution. Plasma albumin concentration fell, and there was a shift of plasma albumin to extravascular sites. Albumin degradation decreased and albumin synthesis decreased even further, leading to a decrease in total exchangeable albumin. The diminished quantity of albumin degraded in the presence of dextran may be related to the lowered plasma albumin concentration, pool size, or both. Precisely the opposite result has been observed with an elevated plasma albumin level and total exchangeable albumin pool (26).

That albumin synthesis was not stimulated, but actually decreased in the presence of low albumin concentration and pool size, indicates that the mechanism responsible for albumin synthesis is not sensitive to low albumin levels per se and suggests that the mechanism might be sensitive to oncotic pressure. The frequently observed fall in albumin concentration accompanying elevations in globulin concentration is in agreement with this concept (27). In the present studies, the decrease in *plasma* oncotic pressure resulting from the decrease in albumin concentration was not compensated for by the increase in oncotic pressure calculated from the measured concentration of circulating dextran. This conclusion is deduced as follows. It has been reported that the osmotic pressure of serum albumin at pH 7.4 and at concentrations less than 5 g per 100 ml obeys van't Hoff's law after correction for the Gibbs-Donnan effect (28, 29). Thus, the observed decrease in plasma albumin concentration of 1.43 g per cent following dextran infusions results in a calculated decrease in the plasma colloid osmotic pressure of 7.6 mm Hg. Using the maximal value observed (2 g per 100 ml) for plasma dextran concentration and assuming that the average

reached values 10.9 to 21.5 per cent greater than those observed with dextran alone. These results are similar to those previously reported after a single dose of albumin-I^{131} (10).

[4] Proteinuria was not observed and the urinary I^{131} activity was not precipitable with trichloroacetic acid.

molecular weight of the circulating dextran was 188,000, the colloid osmotic pressure contributed by dextran alone would be only 2.1 mm Hg. However, the site of albumin production is most likely in direct contact with extracellular fluid rather than with plasma, and the results of these studies indicate that the total extravascular albumin suffered a lesser reduction than total plasma albumin during dextran treatment (Table IV). Unless the interstitial space increased even more than did the plasma volume, these results would indicate that the interstitial concentration of albumin suffered a lesser reduction than did the plasma albumin concentration. Thus it is possible that in certain critical areas adjacent to or within sites of albumin synthesis, the sum of interstitial dextran and albumin could have resulted in a colloid osmotic pressure high enough to inhibit albumin synthesis.[5]

Upon the addition of cortisone, albumin distribution and metabolism were again altered. Plasma volume was nearly doubled and plasma albumin concentrations rose (in four of six rabbits) indicating a return of albumin from extravascular locations to the plasma. The absence of any change in plasma dextran concentration, in spite of a marked increase in plasma volume during combined dextran-cortisone therapy, suggests that cortisone probably mobilized extravascular dextran as well. Cortisone administration has previously been shown to result in increased albumin catabolism (10) and this effect was observed again; however, albumin synthesis increased to a greater extent, resulting in a total exchangeable albumin pool even larger than that observed during the control study.

In the presence of dextran and a low albumin concentration, cortisone administration not only resulted in a more marked increase in albumin synthesis than when it was given without prior dextran therapy, but also caused a greater shift of extravascular albumin to the plasma than had been observed previously in the absence of dextran (10). In addition, cortisone therapy has been reported to cause an increase in extracellular volume (30, 31) even in the absence of weight gain (30). Thus it may be suggested that, following cortisone administration, there might have been a reversal of the elevated extravascular colloid concentration postulated under dextran administration, with a resultant augmented stimulus for albumin production. However, until it becomes possible to measure extracellular colloid concentration, these considerations must remain purely speculative.

TABLE IV
Albumin partition

	Control	Dextran	Dextran + cortisone
Intravascular albumin			
Mean value, g	6.2	4.9	8.1
Change, %		−21.0	+65.4
Extravascular albumin			
Mean value, g	10.1	9.0	10.6
Change, %		−10.7	+17.8

Other factors must also be considered in attempting to interpret the observed changes in albumin metabolism. The depression of albumin synthesis by dextran might have been due to a specific toxic action unrelated to osmotic activity. However, the rapid return to normal following discontinuation of dextran indicates that such action, if it exists, is readily reversible. While no specific mechanism has been identified as responsible for the observed changes in albumin synthesis, it is clear that, in the presence of dextran, a low albumin concentration is not an adequate stimulus for normal albumin synthesis. Addition of cortisone results in a marked stimulation of albumin synthesis.

SUMMARY

1. The effects of daily dextran infusions on the distribution and metabolism of albumin-I^{131} were studied in 12 female rabbits. Cortisone acetate was administered to 6 of the rabbits after prolonged dextran infusions and to 2 rabbits from the onset of dextran administration. Two rabbits were re-examined 6 and 8 weeks after dextran infusions were discontinued, and the effects of saline infusions were studied in 2 additional rabbits.

[5] It is recognized that if a disproportionately large number of smaller dextran molecules were present, then the calculated colloid osmotic pressure due to dextran would be higher than the computed value given above. However, such a change in the colloid osmotic pressure would seem to be applicable to both plasma and interstitial fluid and would not tend to refute the above hypothesis.

2. Under the influence of dextran, total exchangeable albumin was diminished, while plasma volume and total apparent space for albumin increased. The rate of albumin degradation decreased by 22.3 per cent and the rate of albumin synthesis by at least this much in the presence of a lowered plasma albumin concentration.

3. Upon the addition of cortisone, the plasma volume and total apparent space for albumin distribution increased markedly, with a shift of albumin stores from extravascular to intravascular sites. Albumin degradation increased 49 per cent over control values and 91.8 per cent above the value obtained during dextran infusions alone. The increment in albumin synthesis was probably more marked, since the total exchangeable albumin pool increased in the face of the increase in albumin degradation. Albumin synthesis seems unrelated to albumin levels per se.

4. Albumin synthesis may be regulated by the level of oncotic pressure in certain critical locations adjacent to or within sites of albumin synthesis.

ACKNOWLEDGMENTS

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THE EFFECTS OF REDUCED CARDIAC SYMPATHETIC TONE ON MYOCARDIAL FUNCTION *

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Stimuli mediated over cardiac sympathetic nerves increase the work output of the heart (1) and the contractility of the myocardium (2). Such stimuli increase ventricular performance so that more external work is produced at a lower filling pressure (3). Stimuli mediated over the vagus nerves appear to decrease ventricular performance (4). Recent work indicates that sympathetic stimulation increases and that vagal stimulation decreases the vigor of atrial contraction (5). The data from these same experiments show also that the relation between mean atrial pressure and stroke work is determined by atrial as well as ventricular function (5).

When one considers that autonomic stimuli modify ventricular filling time as well as ventricular performance it becomes apparent that the regulation of the cardiac output in the normal subject must involve a very delicate balance between the autonomic and humoral factors that affect the myocardium directly, and all of the factors that influence the availability of blood to the heart. It is not surprising that it has been difficult to show a distinct correlation between mean atrial or end-diastolic pressure and cardiac output in dogs with closed chests (6–9) and in man (10–13).

The experiments to be reported here were carried out in very lightly anesthetized dogs with closed chests and intact circulations. They show that the heart, operating with its rate controlled and in the presence of reduced autonomic tone, increases its output when mean atrial pressure is increased. They also show that cardiac output at a given heart rate and a given atrial pressure is reduced when existing activity of the cardiac sympathetic nerves is reduced.

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METHODS

Mongrel dogs were lightly anesthetized with 100 to 150 mg of thiopental sodium (Pentothal Sodium) and then given gallamine triethiodide (Flaxedil), 5.0 mg per kg intravenously. A cuffed endotracheal tube was inserted immediately through the larynx and connected to a fixed-volume respiratory pump. Both vagus nerves, the left carotid artery and the right external jugular vein at its bifurcation were exposed through three small incisions within the next 10 to 15 minutes. At this point a second small dose of thiopental (about 50 mg) was given and bilateral vagotomies were done. The animal was then turned into the left lateral position and a double-lumen catheter (both openings at the tip) was manipulated through a branch of the external jugular vein under fluoroscopic visualization until its tip lay in the main pulmonary artery. A second catheter with a single lumen and an electrode near the tip was introduced through the other branch of the jugular vein and placed so that its tip lay in the right atrium near the junction with the superior vena cava. The locations of the tips of both catheters were marked carefully during fluoroscopy with the dog supine and lying on the left side. An electrode was attached to the chest wall immediately opposite the tip of the intra-atrial catheter. Statham strain gages were connected to the intra-atrial catheter and to one lumen of the pulmonary arterial catheter. The gages were mounted vertically on the sides of the dog board so that their reference levels coincided with lines formed by the intersection of vertical and horizontal planes passing through the tips of the catheters. The gages were mounted on swivels and kept in the vertical position when the dog board was tilted so that the same relationship between catheter tip and gage would be maintained. Needles were inserted into the left carotid and femoral arteries and the left femoral vein. The needle in the carotid artery was connected through small-bore nylon tubing to the cuvet of a Gilford densitometer. The needle in the femoral artery was connected to a strain gage through a short piece of polyethylene tubing. The needle in the femoral vein was connected to a stopcock and used for intravenous administration of drugs. End-expiratory CO_2 concentration was monitored with a Liston-Becker CO_2 analyzer. The other lumen of the

pulmonary arterial catheter was used for injections of indocyanine green dye. Dye curves were obtained by drawing blood from the left carotid artery through the densitometer with a constant speed pump. Dye curves, end-expiratory CO_2 concentrations and blood pressures were recorded simultaneously with a Sanborn direct-writing oscillograph.

After the needles and catheters were in position, the animals were blindfolded and touched as infrequently as possible. All unnecessary noise was avoided. Ventilation was adjusted so that end-expiratory CO_2 concentration was about 4 per cent. The rate and depth of breathing were not changed after this initial adjustment. Twenty to 30 minutes was allowed for blood pressures and heart rate to become stable after the animal was prepared. This, in addition to the time required for preparation, was ample time for nearly complete disappearance of the thiopental effects (14, 15). Injections of indocyanine green dye were then made into the pulmonary artery at 5- to 10-minute intervals. The resulting dye curves were recorded and examined carefully. After appearance time, peak concentration time and general configuration became stable, the dog was tilted into the 20° head-down position. Four to 7 minutes was allowed for blood pressure and heart rate to become stable again, then two dye curves were obtained and the dog board was returned to the horizontal position. After restabilization of the heart rate and blood pressure, 15.0 mg of the ganglionic blocking agent, trimethidinium methosulphate (Ostensin, Wy-1395) (16–19), was given intravenously. Ten to 15 minutes more was allowed for blood pressures and heart rate to level off. Observations were then made in the supine and head-down positions in the same manner as before trimethidinium. After the second dye curve was obtained with the dog tilted, the heart rate was restored as nearly as possible to that existing before the drug, by stimulating the heart with a 2 to 4 msec square wave of 2 to 5 v through the intra-atrial and chest wall electrodes. Two more curves were obtained in quick succession at the restored heart rate with the animal head-down.

Electrically integrated mean right atrial and pulmonary arterial blood pressures were recorded throughout each dye curve. Mean systemic arterial pressure was recorded immediately before and after obtaining each curve. Phasic arterial pressure was registered during the curve so that heart rate could be counted. Fifty ml of blood was taken at the beginning of the experiment for calibration of the densitometer. The blood required for inscription of each curve was returned to the dog immediately by reversing the motor on the withdrawal pump. Dye solutions were made up before the experiment, and a small amount of blood was added so that the dye in the injectate would be bound to plasma. This prevents the change in optical density with time that would occur in an aqueous solution. Three-point calibration curves were used in each experiment. Approximately 2.5 mg of dye was used for injection. Background dye in the blood was "zeroed" out with the variable density wedge on the densitometer before each injection. The numerical setting of the wedge corresponding to the background was recorded for each determination. The amount of background dye could be estimated from this information, which made it possible to allow an appropriate amount of time between groups of injections to insure against excessive accumulations of the dye in the blood.

The values to be reported are the final observations obtained in the supine and head-down positions. The values reported with pacing of the heart are the first observations made after the heart rate nearest the control rate was established. All systemic arterial pressures are referred to right atrial level. Pulmonary arterial and atrial pressures are referred to the tips of the catheters. The strain gages were calibrated with a water manometer graduated in millimeters of mercury. Zero suppression circuits and high sensitivities were used so that small changes in right atrial and pulmonary arterial

TABLE I

Responses to increased right atrial pressure before administration of a ganglionic blocking agent *

Dog	Wt	RA_m		PA_m		SA_m		HR		CO		SV		RVMW		RVSW		LVMW		LVSW	
		S	HD	S	HD	S	HD	S	HD	S	HD	S	HD	S	HD	S	HD	S	HD	S	HD
	kg	*mm Hg*		*mm Hg*		*mm Hg*		*beats/min*		*L/min*		*ml*		*units*		*units*		*units*		*units*	
1	23.9	4.5	5.5	15.5	18.0	153	171	162	168	3.0	3.1	19	18	33	39	0.20	0.23	438	505	2.7	3.0
2	18.3	2.7	3.9	13.0	12.5	148	156	248	244	2.3	2.4	9	10	24	21	0.10	0.09	328	359	1.3	1.5
3	17.5	0.5	1.3	13.8	16.3	150	159	212	216	2.6	2.7	12	13	34	41	0.16	0.19	382	419	1.8	1.9
4	19.0	3.1	5.4	16.4	18.5	137	135	224	220	2.4	2.4	11	11	32	31	0.14	0.14	315	305	1.4	1.4
5	16.0	2.5	3.2	16.0	18.0	155	165	152	152	2.4	2.4	16	16	32	33	0.21	0.22	360	382	2.4	2.5
6	20.5	5.1	6.5	14.8	16.6	137	165	174	180	2.3	2.4	13	13	22	24	0.13	0.13	298	374	1.7	2.1
7	21.7	6.0	8.0	16.5	19.2	158	153	180	188	2.8	2.9	16	15	29	32	0.16	0.17	419	413	2.3	2.2
8	17.5	2.3	4.8	12.3	18.0	132	137	200	180	2.2	2.3	11	13	22	30	0.11	0.17	280	296	1.4	1.6
9	21.8	3.2	3.8	8.0	12.4	123	111	228	208	2.6	2.8	11	13	15	24	0.07	0.12	305	293	1.3	1.4
10	17.0	4.1	5.3	13.3	14.2	127	128	208	200	2.4	2.3	12	12	22	20	0.11	0.10	289	276	1.4	1.4

* RA_m, right atrial mean pressure; PA_m, pulmonary arterial mean pressure; SA_m, systemic arterial mean pressure; HR, heart rate; CO, cardiac output; SV, stroke volume; RVMW, right ventricular minute work index; RVSW, right ventricular stroke work index; LVMW, left ventricular minute work index; LVSW, left ventricular stroke work index; S, supine position; HD, head-down position.

mean pressures would cause large deflections on the recording paper. In most cases the sensitivity of the transducer-amplifier system was such that a pressure of 1 mm Hg caused a deflection of 1 cm. The respiratory variation in pressure was very constant so that directional changes in right atrial and pulmonary arterial pressure could be recorded with exactness. Mean pressures were obtained by electrical integration of the output of the strain gages. Because of the sensitivity of the system and the constancy of ventilation, it was felt that mean atrial and pulmonary arterial pressures should be read in millimeters of Hg, using one decimal figure. Cardiac output was calculated by the Stewart-Hamilton method. Right ventricular minute work index was calculated according to the formula $(PA_m - RA_m) \times CO$. Left ventricular minute work index was calculated according to the formula $(SA_m - LA_m) \times CO$. Stroke work index was equal to minute work index divided by heart rate. All indices of cardiac work are expressed in arbitrary units. PA_m is pulmonary arterial mean pressure in millimeters of Hg. RA_m is right atrial mean pressure in millimeters of Hg. CO is cardiac output in liters per minute. SA_m is systemic arterial mean pressure in millimeters of Hg, and LA_m is an approximation of left atrial mean pressure, also in millimeters of Hg. Left atrial pressure was not measured. An arbitrary approximation of the left atrial pressure was taken as right atrial pressure plus 2.5 mm Hg.

The data were analyzed according to methods described by Fisher (20).

RESULTS

The data are recorded in Tables I and II. The responses to tilting are compared in Tables III and IV.

Right atrial pressure. Right atrial mean pressure increased when the dogs were tilted into the head-down position before ganglionic blockade. The increase averaged 1.4 mm Hg. The pressure in the supine position did not change consistently after ganglionic blockade, but tilting again led to an average increase of 1.4 mm Hg. The difference between the responses to tilting before and after trimethidinium (Table III) was not significant ($p < 0.9$). There was essentially no change in right atrial pressure (Table IV) when heart rate was restored electrically with the animal head-down.

In these experiments, changes in right atrial pressure (measured with respect to atmospheric pressure) are considered to reflect changes in transmural right atrial pressure. This was confirmed in three dogs by measuring atrial pressure with reference to intraesophageal pressure (which reflects intrapleural pressure) during tilting.

TABLE II

Responses to increased atrial pressure after administration of a ganglionic blocking agent *

Dog	RA_m			PA_m			SA_m			HR			CO			SV			RVMW			RVSW			LVMW			LVSW		
	S	HD	HDR	S	HD	HDR	S	HD	HDR	S	HD	HDR	S	HD	HDR	S	HD	HDR	S	HD	HDR	S	HD	HDR	S	HD	HDR	S	HD	HDR
	mm Hg			*mm Hg*			*mm Hg*			*beats/min*			*L/min*			*ml*			*units*			*units*			*units*			*units*		
1	4.5	5.5	5.5	12.5	16.2	15.5	123	162	154	150	144	162	2.2	2.7	2.6	15	19	16	18	29	26	0.12	0.20	0.16	257	416	380	1.7	2.9	2.3
2	0.7	2.5	2.0	4.8	6.5	5.5	75	128	96	144	140	196	1.1	1.3	1.2	8	9	6	5	5	4	0.03	0.04	0.02	79	160	110	0.5	1.1	0.6
3	0.3	1.0	1.0	8.3	12.3	13.0	95	127	132	136	128	208	1.4	1.5	1.5	10	12	12	11	17	18	0.08	0.13	0.09	129	185	193	0.9	1.4	0.9
4	1.0	2.9	2.9	13.5	12.5	13.7	74	95	101	180	168	204	1.1	1.4	1.5	6	8	7	14	13	16	0.08	0.08	0.08	78	125	143	0.4	0.7	0.7
5	2.0	2.6	2.6	9.5	15.0	15.0	98	118	122	132	132	168	1.5	1.7	1.8	11	13	11	11	21	22	0.08	0.16	0.13	140	192	210	1.1	1.5	1.3
6	5.1	6.5	6.5	9.6	12.4	12.4	112	140	105	132	132	168	1.5	1.8	1.9	11	14	11	7	11	11	0.05	0.08	0.07	157	236	182	1.2	1.8	1.1
7	7.1	9.2	9.2	12.6	17.5	18.0	127	123	118	144	144	172	2.1	2.4	2.5	15	17	15	12	20	22	0.08	0.14	0.13	247	267	266	1.7	1.9	1.5
8	3.8	5.5	5.3	7.8	11.8	12.5	103	110	112	128	120	188	1.3	1.6	1.7	10	13	9	5	10	12	0.04	0.08	0.06	126	163	177	1.0	1.4	0.9
9	3.4	4.7	4.6	6.5	10.0	10.7	90	110	103	144	144	192	1.8	2.5	2.4	13	17	13	6	13	15	0.04	0.09	0.08	151	257	223	1.0	1.8	1.2
10	3.5	5.0	4.8	8.5	10.2	11.5	52	100	100	140	128	204	0.9	1.4	1.4	6	11	7	5	7	9	0.04	0.04	0.04	41	130	130	0.3	1.0	0.6

* See footnote to Table I. HDR, head-down position with heart rate restored.

TABLE III

Comparison of responses to increased right atrial pressure before and after administration of a ganglionic blocking agent *

	RA_m		PA_m		SA_m		HR		CO		SV		RVMW		RVSW		LVMW		LVSW	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
	mm Hg		*mm Hg*		*mm Hg*		*beats/min*		*L/min*		*ml*		*units*		*units*		*units*		*units*	
Mean	1.37	1.40	2.41	3.08	6.0	26.4	3.2	5.0	0.07	0.34	0.4	2.8	3.0	5.0	0.017	0.040	20.8	60.6	0.13	0.47
Standard error	0.213	0.170	0.527	0.596	3.64	5.56	3.24	1.56	0.027	0.057	0.34	0.39	1.37	1.18	0.0077	0.0089	10.30	13.67	0.047	0.065
Probability	<0.001	<0.001	<0.01	<0.001	<0.2	<0.01	<0.4	<0.02	<0.1	<0.001	<0.2	<0.001	<0.1	<0.01	<0.1	<0.01	<0.1	<0.01	<0.05	<0.001
Mean difference (A vs B)	0.03		0.67		20.4		1.8		0.27		2.4		2.0		0.023		39.8		0.34	
Standard error	0.139		0.633		5.48		3.29		0.058		0.52		1.49		0.0071		10.13		0.075	
Probability	<0.9		<0.4		<0.01		<0.6		<0.01		<0.01		<0.3		<0.02		<0.01		<0.01	

* See footnote to Table I. A is the mean difference between values observed in the head-down and supine positions before ganglionic blockade. B is the mean difference after ganglionic blockade.

TABLE IV

Comparison of measurements made before and after restoration of the heart rate *

	RA_m		PA_m		SA_m		HR		CO		SV		RVMW		RVSW		LVMW		LVSW	
	HD	HDR	HD	HDR	HD	HDR	HD	HDR	HD	HDR	HD	HDR	HD	HDR	HD	HDR	HD	HDR	HD	HDR
	mm Hg		*mm Hg*		*mm Hg*		*beats/min*		*L/min*		*ml*		*units*		*units*		*units*		*units*	
Mean	4.5	4.4	12.4	12.8	121	114	138	186	1.8	1.9	13	11	15	16	0.10	0.09	213	201	1.6	1.1
Mean difference (HD vs HDR)	0.10		0.34		7.0		48.2		0.02		2.6		0.9		0.018		11.7		0.44	
Standard error	0.055		0.240		4.69		6.67		0.028		0.42		0.57		0.0044		9.99		0.065	
Probability	<0.2		<0.2		<0.2		<0.001		<0.5		<0.001		<0.2		<0.01		<0.3		<0.001	

* See footnotes to Tables I and II. These observations were made in the head-down position after administration of a ganglionic blocking agent.

The close relationship between right atrial mean pressure and transmural pressure is not surprising. The dogs were ventilated with a respiratory pump that kept ventilatory rate and depth constant. Skeletal muscle relaxation was pronounced because of the gallamine, and tilting the dogs into the 20° head-down position led to very little shift in abdominal contents. All of these factors tended to stabilize intrapleural pressure in this preparation.

Pulmonary arterial pressure. Pulmonary arterial mean pressure increased regularly with tilting. The increase averaged 2.4 mm Hg before and 3.1 mm Hg after trimethidinium. Pulmonary arterial pressure in the supine position was always reduced following ganglionic blockade. The response to tilting, however, was not altered significantly (Table III) by the blocking drug ($p < 0.4$). Increasing heart rate in the head-down position had an insignificant effect on the pressure ($p < 0.2$).

Systemic arterial pressure. Femoral arterial mean pressure did not change appreciably with tilting before trimethidinium. After the drug, tilting led to an increase which averaged 26.4 mm Hg. The increase in arterial pressure with tilting was significantly greater (Table III) after trimethidinium ($p < 0.01$). Increasing heart rate in the head-down position had an insignificant effect (Table IV) on the pressure ($p < 0.2$).

Heart rate. The gallamine-prepared dog has a rapid heart rate. This is attributed to the vagolytic action of the drug (21) and in these experiments to the vagotomy. The sharp reduction in resting rate with trimethidinium is attributed to reduction in cardiac sympathetic tone in the presence of the vagotomy. The heart rate did not change much with tilting, and the difference between responses to tilting before and after ganglionic blockade (Table III) was not significant ($p < 0.6$). It was not possible in each instance to restore the rate exactly to the resting level by electrical stimulation. Failure to achieve the ideal rate occurred in animals whose resting rate was very rapid. The restored rate is the rate nearest to the resting value without irregularities in rhythm. The restored rate averaged 186 beats per minute, 13 less than the control.

Cardiac output and stroke volume. Cardiac output increased from an average of 2.5 L per minute in the supine position to only 2.6 L with tilting before administration of the blocking drug (Table I). Output in the supine position fell to an average of 1.5 L per minute after trimethidinium but increased to an average of 1.8 L with tilting (Table II). Although the resting output was lower, the response to tilting was appreciably greater (Table III) after ganglionic blockade ($p < 0.01$). Restoration of the heart rate with the dogs tilted had no effect on cardiac output.

Stroke volume (Table III) was essentially unchanged with tilting before trimethidinium. It increased an average of 2.8 ml with tilting after the drug ($p < 0.001$), and fell by an average of 3.2 ml with restoration of the heart rate in the head-down position ($p < 0.001$). The reduction in cardiac output with ganglionic blockade appears to be caused primarily by a fall in stroke volume rather than by a decrease in rate, since output remained lower than the control level even after the rate was restored.

Cardiac work. It is customary to describe the performance of the ventricles in terms of the external work they do. External work is represented as the product of stroke volume or minute volume and mean arterial pressure minus mean atrial pressure. These indices have been calculated and included in the tables. The values for minute work and stroke work tend to parallel the values for cardiac output and stroke volume.

DISCUSSION

Gallamine (21) is a muscle relaxant similar to curare. It does not, however, have the ganglionic-blocking and histamine-releasing properties of the latter drug. Blood pressure is not depressed and consciousness and sensory function apparently are not disturbed. Cardiovascular responses to stimuli mediated over the autonomic nervous system are considered to be predominantly sympathetic in the preparation used in these experiments because of the vagotomy and the selective vagolytic action (21) of the gallamine. These dogs appear to have highly functional sympathetic pathways. Their cardiovascular responses to many stimuli are similar to those that might be observed in unanesthetized dogs. Light touch or unusual sounds within the room cause marked fluctuations in heart rate and blood pressure. Cardiac output has been observed to double or triple the resting level

following an unusual noise or mildly painful stimulus.

Recent studies by Sarnoff (*22*) and others (3–5) indicate that changes in the performance of the heart muscle are related to the degree of sympathetic stimulation. At a constant heart rate, increased sympathetic stimulation of the myocardium causes the heart to do more work at a lower filling pressure (3). It appears that withdrawal of sympathetic tone from the heart might cause it to do less work at a higher filling pressure. The results of the experiments reported here fit these concepts of the regulation of myocardial function. In the presence of intact sympathetic pathways, the heart failed to increase its work output significantly when atrial pressure increased. After sympathetic tone was removed by ganglionic blockade, the heart responded more clearly to an increase in filling pressure. In the first instance, failure of output to increase with the increase in filling pressure may mean that sympathetic tone to the myocardium is withdrawn, possibly by some reflex mechanism. The net effect of such a reflex might be a new relationship between atrial pressure and ventricular function (higher pressure and reduced function) which results in no change in output or external work of the heart. In the second situation, pharmacologic denervation appeared to remove the sympathetic influence which normally alters myocardial performance. It became impossible for the effect of the increased filling pressure to be modified by a reduction in the functional characteristics of the heart muscle. The result of such denervation is a heart that increases its output with an increase in filling pressure. Such behavior resembles that of a heart-lung preparation.

The administration of trimethidinium led to reduction in the supine levels of cardiac output, stroke volume and ventricular work. Significant increase in output and work were noted when filling pressure was increased. Despite these increases, the levels seen before ganglionic blockade were not achieved. Even restoration of the heart rate caused no further increase in cardiac output or minute work of the ventricles. Recent studies suggest that the reduced cardiac output associated with ganglionic blocking drugs is not the result of myocardial depression (23). Some believe that the fall in output is caused primarily by venous pooling (24, 25). Beck (*26*), however, has demonstrated clearly in intact dogs that cardiac output falls with hexamethonium administration even if atrial pressure is held constant. The experiments reported here support those of Beck and indicate that the fall in output after administration of ganglionic blocking drugs may be attributed, at least in part, to changes in myocardial function. The reduced cardiac output following ganglionic blockade when filling pressure is controlled is caused primarily by the fall in stroke volume, since restoring heart rate to the control level leaves the minute volume unchanged.

In these experiments the fall in cardiac output and work is attributed to a reduction in the inflow of sympathetic impulses to the myocardium. The possibility remains that the decrease in cardiac function might be related to a direct depressant action of trimethidinium on the heart muscle. We have not been able to find evidence in the literature that would suggest that ganglionic blocking drugs depress the myocardium directly. Beck (*26*) also has been unable to find such evidence. He cites the work of Acheson and Moe (*27*), who found that tetraethyl ammonium increases the output of the heart-lung preparation. Lee and Shideman (*28*) found that the positive inotropic effect on the myocardium, produced by ganglionic stimulating drugs, could be blocked by ganglionic blocking agents. The available evidence indicates that the myocardial "depressant" effects of ganglionic blocking agents are related to blockade of nervous impulses and not to a direct action on the muscle.

SUMMARY

The ventricular responses to the increased filling pressure produced by tilting into the head-down position were studied in 10 vagotomized dogs treated with gallamine. Before administration of a ganglionic blocking agent there were only slight changes in heart rate, cardiac output, stroke volume and external work of the ventricles in response to the rise in pressure. After cardiac sympathetic tone was reduced by administration of a ganglionic blocking drug (trimethidinium) heart rate, cardiac output, stroke volume and ventricular work fell. Increasing atrial pressure

caused significant increases in output and work, but the levels present before trimethidinium were not achieved. Restoration of the heart rate by electrical stimulation of the atrium with the animals in the head-down position caused no further increase in cardiac output or work.

It is suggested that increased atrial pressure in the presence of an intact sympathetic nervous system causes little change in the work output of the heart because of reflex withdrawal of sympathetic tone to the myocardium. Pharmacologic denervation of the heart appears to remove this sympathetic influence which might otherwise modify the effect of an increased filling pressure. The denervated heart responds more clearly to an increase in filling pressure. In this respect its performance resembles that of a heart-lung preparation.

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THE INFLUENCE OF INTRACARDIAC BARORECEPTORS ON VENOUS RETURN, SYSTEMIC VASCULAR VOLUME AND PERIPHERAL RESISTANCE

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The presence of baroreceptors in the walls of the cardiac chambers and the pulmonary vascular bed is now well established, and their function in the reflex control of the circulation has been the subject of a number of investigations (1–11). Action potentials have been recorded from afferent fibers originating in the heart and lungs (1, 2), and elevation of pressures within the ventricular chambers (3–9) and pulmonary vascular bed (4, 10, 11) has resulted in bradycardia (3–10), a decline in systemic arterial pressure (4–9, 11), and either apnea (5, 10) or hyperpnea (4, 11). In a previous report from this laboratory, evidence was presented that the carotid baroreceptors reflexly influence total venous return and systemic vascular volume (12). The present investigations were undertaken to determine whether or not these hemodynamic parameters are also modified by activation of intracardiac baroreceptors. Studies to localize these receptors within the heart were also carried out.

METHODS

Seventeen dogs weighing from 15.1 to 23.0 kg, average, 17.2, were anesthetized with morphine (2 mg per kg), chloralose (48 mg per kg), and urethan (480 mg per kg). Following tracheal intubation, a bilateral thoracotomy was performed through the fourth intercostal space and the sternum was transected. Heparin (2 mg per kg) was then administered intravenously. The femoral artery was cannulated, large cannulae were inserted into each vena cava through the right atrium, and total body perfusion was carried out with a pump-oxygenator system as described elsewhere (13). Briefly, blood was drained by gravity from the venae cavae into a rotating disc oxygenator and was returned to the femoral artery by means of a roller pump through a recording rotameter (14) which continuously metered the perfusion rate. Systemic perfusion rates ranged from 73 to 109 and averaged 96 ml per kg. Blood temperature was maintained between 34° and 37° C by means of a heating coil around the oxygenator. In 10 dogs, measurements of alterations in the volume of blood in the extracorporeal circuit were carried out. Changes in the level of blood in the oxygenator were detected by means of sensing electrodes (15), and blood was added to or removed from a calibrated reservoir so that the volume of blood in the oxygenator remained constant. Alterations in extracorporeal blood volume, which reflected reciprocal changes in the intravascular blood volume, were measured by recording the level in the calibrated reservoir at 1 minute intervals. In 3 animals, intravascular blood was maintained at a constant volume by increasing or decreasing the arterial pump output; with this maneuver the effect of any intervention on venous return could be determined (16). For example, when venous return to the oxygenator diminished, the pump output was decreased manually at a rate sufficient to maintain the volume of the extracorporeal circuit constant.

Pressures were measured in the aorta and the inferior vena cava through catheters inserted into small branches of the femoral artery and vein; pressures in the right and left ventricles were obtained by means of catheters inserted directly through the ventricular walls, and in the left atrium by means of a catheter introduced through a pulmonary vein. All pressures were measured with Statham pressure transducers and were recorded continuously, together with the systemic perfusion rate, on a multichannel oscillograph. Since the systemic perfusion rate was held constant throughout each experiment, any change in arterial pressure reflected an identical change in peripheral resistance.

In order to study the reflex effects of changes in intracardiac pressure, two types of preparations were employed. In the first preparation, utilized in Dogs 14, 15 and 16 (Table I), the aorta was occluded above the coronary arteries, and blood was supplied to the heart through a line which originated between the pump and the flowmeter. Total systemic perfusion rate was held constant. In these experiments, blood entered the left ventricle through a cannula which had been inserted through the apex. The rate of blood flow into the heart, which determined the pressure in the left side of the heart, was regulated by means of a screw clamp on the inflow line. This blood was pumped by the left ventricle into the aortic segment proximal to the occlusive clamp, thence through the coronary circulation, and was returned to the pump oxygenator by means of a large drainage tube placed in the right ventricle via the azygos vein and right atrium. Although this preparation was satisfactory for studying the effects of changes in intracardiac pressures on peripheral resistance, it was unsuitable for the measurement of alterations of intravascular blood volume and

venous return; when the blood flow to the heart was increased, a significant volume was necessarily diverted from the extracorporeal circuit into the cardiac chambers and pulmonary circulation.

Accordingly, in the second preparation, blood was circulated through the heart by utilizing a separate circuit, entirely isolated from the pump-oxygenator system (Figure 1). Oxygenated blood drained from a reservoir through a cannulated pulmonary vein and into the left side of the heart. In addition to the drainage cannula placed in the right ventricle, a tube was inserted into a segmental pulmonary artery to assure complete emptying of the right heart and pulmonary artery; the blood from these two drainage tubes was collected in an accessory reservoir. Circulation through the lungs was prevented by mass ligation of each hilum. Alterations in pressure in the left side of the heart could be induced by varying the inflow from the reservoir while the ascending aorta was kept clamped. When the inflow of blood to the pulmonary vein was increased, left heart pressures rose, since the only egress of blood was through the coronary bed. Pressure in the right side of the heart could be modified by varying the resistance in the right ventricular and pulmonary arterial drainage lines, since clamping of the lines resulted in a "damming up" of blood in the right side of the heart. When left ventricular systolic pressure was maintained at a level exceeding approximately 70 mm Hg, the heart continued to beat satisfactorily for several hours. During each experiment, control pressures, extracorporeal blood volume and heart rate were determined for at least 4 minutes, and following each intervention, measurements were recorded continuously for another 4 to 8 minutes. This preparation was employed in 14 dogs, two of which (7 and 8, Table I) had been subjected to splenectomy several weeks prior to study.

In an effort to define the activity of the intracardiac receptors more precisely, bilateral mass ligation of the carotid bulb was performed in the 3 dogs in which the first preparation was employed and in 10 of the 14 experiments utilizing the second preparation. In 9 of the 10 dogs in the latter group, the aortic baroreceptors were

TABLE I

Hemodynamic effects of changes in intracardiac pressures

Intervention	Dog no.	Wt	LV press. A	LV press. B	LA press. A	LA press. B	RV press. A	RV press. B	Ao press. A	Ao press. B	Δ B.V.	Δ S.F.	HR A	HR B
		kg	*mm Hg*		*cm H₂O*		*mm Hg*		*mm Hg*		*ml*	*ml/min*		
	1+	19.0	70/6	200/17	8	23	25/3	60/20	66	40	+160		126	120
			75/0	210/10	10	22	25/2	75/15	75	50		−220		
			210/10	65/0	22	10	75/15	20/5	50	69		+ 70		
	2+	16.6	75/1	220/15	6	14	12/9	85/25	120	90			96	102
	3+	19.0	68/3	190/5	6	20	2/0	60/5	84	60		−540		
Left and right			190/5	75/5	20	6	60/5	2/0	60	74		+260		
heart pressures	4+	23.0	77/5	235/22	6	25	1/0	60/10	67	50		−590	120	120
altered			235/30	60/5	25	5	60/10	1/0	58	68		+195	120	132
			100/8	225/40	8	40	1/0	60/10	63	52	+110		114	114
	5	18.3	73/7	225/20	7	30	1/0	70/18	63	50	+170			
	6	17.6	70/6	180/20	10	30	3/0	75/17	74	63	+135		126	114
	7	16.5	95/5	230/30	5	35	5/0	80/13	67	50	+ 90		120	114
	8	15.1	92/8	230/35	9	40	1/0	65/15	78	66	+110		102	102
	9+	20.5	75/10	115/30	8	40	1/0	5/0	68	45			118	108
	10+	16.7	80/6	200/30	7	40	2/0	2/0	84	65	+ 90			
	11+	15.3	110/5	225/30	5	47	5/0	10/0	102	70	+ 40		108	108
	12+	15.5	85/3	225/12	3	16	5/0	5/0	110	66			84	78
Left heart			200/22	80/2	33	5	5/0	7/0	90	115			111	111
pressure only	13+	19.5	80/5	240/35	5	35	10/0	10/0	112	90	+120			
altered			240/35	55/5	35	3	10/0	10/0	90	97			105	120
	14∓	12.2	57/3	175/2	5	7			135	105				
	15∓	16.2	65/0	177/4	0	4			89	75				
	16∓	15.6	75/0	170/3	0	0			93	78				
	17∓	15.4	80/0	175/3	2	6	20/3	22/3	113	75	+170		120	120
	5		85/11	95/13	9	11	1/0	60/11	67	63			132	120
Right heart	6		105/6	110/0	8	8	1/0	73/14	65	62			132	144
pressure only	11		137/12	137/10	12	11	1/0	55/8	88	88			120	116
altered	12		80/2	75/5	5	5	7/0	85/15	112	107			99	111
	13		100/9	100/8	9	9	10/5	65/11	102	99			132	126

* LV = left ventricular pressure; LA = mean left atrial pressure; RV = right ventricular pressure; Ao = mean aortic pressure; HR = Heart rate in beats per minute. Columns A represent observations during the control period; B represent the observations made following alteration of intracardiac pressure. Δ B.V. = the change in systemic vascular volume; Δ S.F. = the change in systemic flow; + = carotid and aortic receptors denervated; ∓ = carotid receptors only denervated.

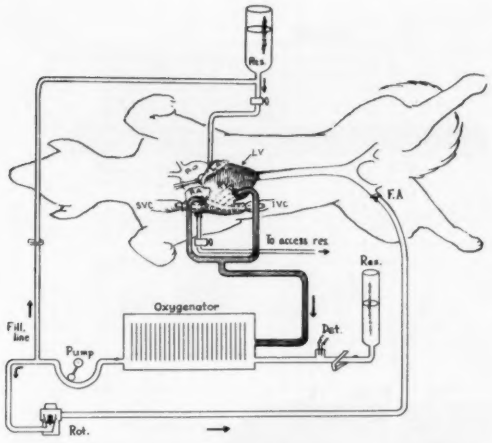

FIG. 1. SCHEMATIC REPRESENTATION OF THE EXTRACORPOREAL CIRCUIT EMPLOYED. Blood drains by gravity from the superior vena cava (SVC) and the inferior vena cava (IVC) into the oxygenator. The aorta (Ao.) is cross-clamped above the coronary arteries. The level of blood in the oxygenator is sensed by the detecting electrodes (Det.) and blood is added to or withdrawn from the calibrated reservoir (Res.) at the lower right-hand corner. Oxygenated blood is pumped through a rotameter (Rot.) into the femoral artery (FA). Intermittently, a clamp on the filling line (Fill. line) is opened to permit replenishment of the top reservoir (Res.) with oxygenated blood.

also removed by stripping the adventitia from the anterior surface of the aorta between the brachiocephalic and subclavian arteries (Table I).

At the conclusion of 6 experiments, the line through which blood entered the left atrium from the reservoir was occluded, resulting in anoxic cardiac arrest or ventricular fibrillation. Alterations in arterial pressure were recorded continuously for several minutes thereafter, while systemic perfusion rate was maintained constant.

RESULTS

I. Alterations in systemic blood volume. In all ten of the dogs in which blood volume alterations were measured (Table I), elevation of intracardiac pressure resulted in a decline in the extracorporeal volume and, therefore, an augmentation of intravascular blood volume, which ranged from 40 to 170 ml and averaged 120 ml. A tracing from an experiment of this type is reproduced in Figure 2. In six of these animals (Dogs 1, 4–8), the pressures in the right side of the heart, the left atrium and the left ventricle were elevated, and in four (Dogs 10, 11, 13, 17), the pressure elevation was confined to the left side of the heart, while right heart pressures were not altered. The increments of intravascular volume observed in these two groups of experiments were similar. The changes in blood volume persisted for the entire period that the intracardiac pressure remained elevated, which in some experiments was up to 15 minutes.

II. Alterations in venous return. In Dogs 1, 3 and 4 the pressures in both the right and left sides of the heart were elevated. In order to maintain the extracorporeal, and therefore the intravascular, blood volume constant, it was necessary to diminish the output of the pump by 590, 540, and 220 ml per minute; this represented an average decrease of 25.3 per cent from the control systemic perfusion rates. When intracardiac pressures were lowered to their previous levels, venous return increased but did not return to the control levels (Figure 3, Table I).

III. Alterations in systemic vascular resistance. In Dogs 1–8, left ventricular pressure was elevated from average values of 78/5 to 216/21 mm Hg; mean left atrial pressure rose from an average of 7 to an average of 25 cm H_2O, and right ventricular pressure rose from average values of 6/1 to 69/15 mm Hg. These pressure alterations resulted in a decline in systemic vascular resistance which was maximal approximately 1 minute later. These maximal changes in systemic vascular resistance ranged from 10 to 39 per cent and averaged 24 per cent of the control resistance levels. Subsequently, the arterial pressure returned partially toward control levels (Figures 2 and 4). This return was less prominent in those dogs which had undergone sinoaortic denervation.

In five experiments (Dogs 9–13) right ventricular pressure was maintained constant at a low level (Table I), while left ventricular pressure was elevated from average values of 86/6 to 203/26 mm Hg, and mean left atrial pressure rose from an average of 6 to an average of 36 cm H_2O. This again resulted in a decline in systemic vascular resistance which ranged from 20 to 40 per cent and averaged 30 per cent of the control resistance levels.

In four experiments (Dogs 14–17) left ventricular systolic pressure alone was elevated. Pressure in this chamber rose from average values of 69/1 to 175/3 mm Hg, while left atrial

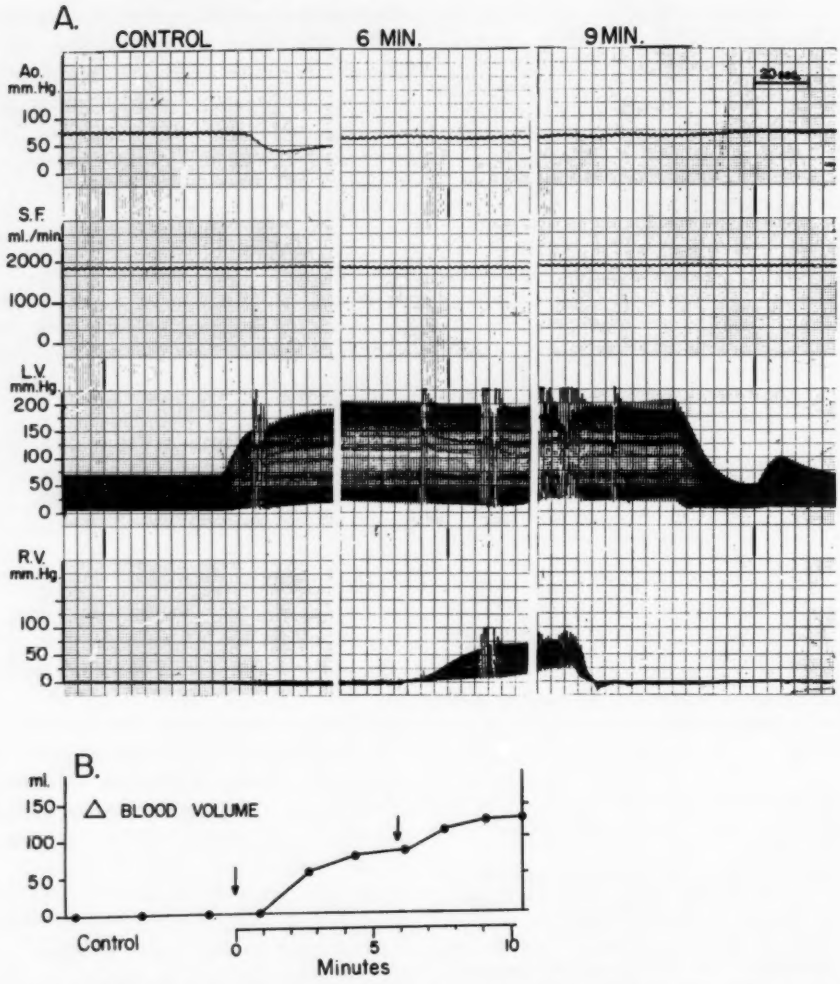

FIG. 2. DOG 6. A. RECORDINGS, FROM ABOVE DOWNWARD, OF PRESSURE IN THE AORTA (AO.), SYSTEMIC FLOW (S.F.), PRESSURES IN THE LEFT VENTRICLE (LV) AND RIGHT VENTRICLE (RV). In the panel on the left, left ventricular pressure was elevated. Six minutes later, in the center panel, right ventricular pressure was also elevated. In the panel on the right, ventricular pressures were lowered.

B. THE ALTERATIONS IN SYSTEMIC VASCULAR VOLUME WHICH OCCURRED SIMULTANEOUSLY ARE DEPICTED. The vertical arrows indicate the points in time at which left and then right ventricular pressures were elevated.

pressure remained low and changed little or not at all. In all of these dogs the right ventricle was decompressed with a large drainage cannula, and in the one dog in which right ventricular pressure was measured, it did not rise. These elevations in left ventricular systolic pressure alone resulted in a decline in systemic vascular resistance which ranged from 16 to 38 per cent (average, 23) of the control resistance levels (Figure 4). In two other experiments when left ventricular systolic pressure was elevated from levels below 70 mm Hg to systolic pressures of 131 and 150 mm Hg, systemic vascular resistance declined 9.6 and 11.8 per cent.

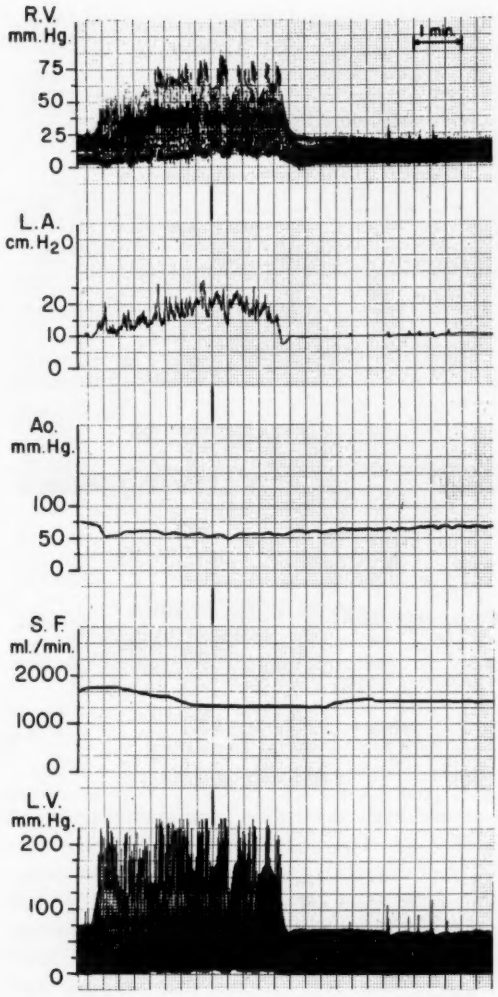

FIG. 3. DOG 1. THE EFFECTS OF ELEVATIONS OF PRESSURES IN THE RIGHT VENTRICLE (RV), LEFT ATRIUM (LA) AND LEFT VENTRICLE (LV), ACCOMPANIED BY EXTRASYSTOLES, ON TOTAL VENOUS RETURN, AS MEASURED BY ALTERING SYSTEMIC PERFUSION RATE (S.F.). The latter declined after intracardiac pressures were elevated and returned toward the control levels following the reduction of these pressures.

In five experiments (Dogs 5, 6, 11–13) right ventricular pressure was elevated from average values of 4/1 to 68/12 mm Hg, while left atrial and left ventricular pressures were maintained at relatively constant levels. Peripheral resistance declined by only 0 to 5 per cent, with an average fall of 3 per cent from control levels (Figure 5).

In the entire series of experiments it was observed that the occurrence of ventricular extrasystoles was accompanied by a brief but striking decline in systemic vascular resistance (Figures 3 and 5A).

IV. Alterations in heart rate. The effects of the elevations in intracardiac pressures on heart rate were variable and slight (Table I). In seven experiments the rate declined by more than 5 beats per minute; in five experiments it rose by

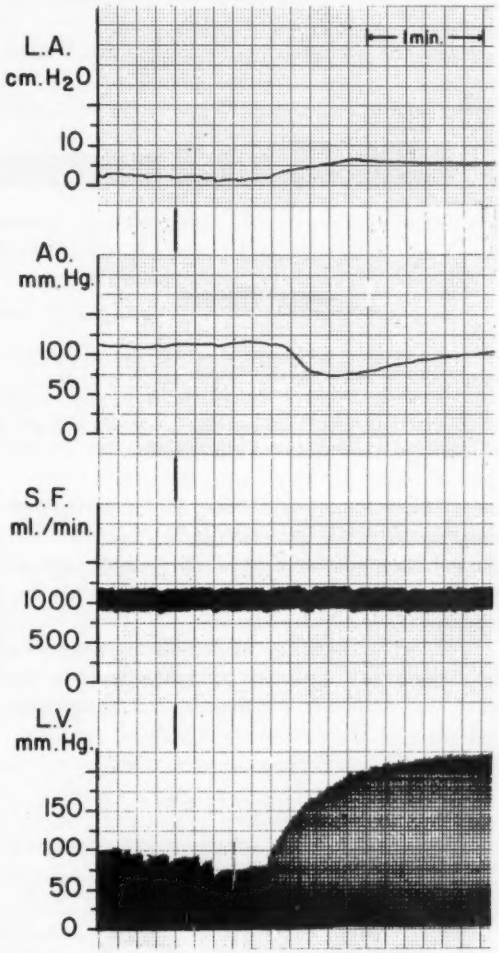

FIG. 4. DOG 17. ALTERATIONS IN AORTIC PRESSURE (AO.) AT A CONSTANT SYSTEMIC FLOW (S.F.) FOLLOWING ELEVATION OF LEFT VENTRICULAR (LV) SYSTOLIC PRESSURE, ACCOMPANIED BY A MINIMAL ELEVATION OF LEFT VENTRICULAR DIASTOLIC AND MEAN LEFT ATRIAL (LA) PRESSURE.

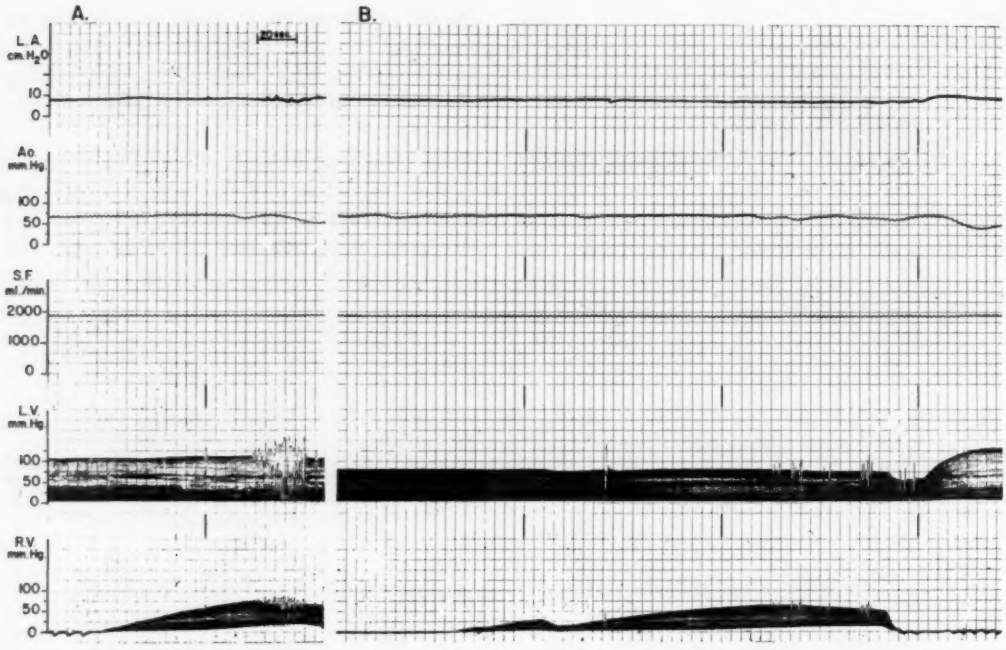

FIG. 5. DOG 6. A. MINIMAL ALERATIONS IN AORTIC PRESSURE ACCOMPANYING ELEVATION OF RIGHT VENTRICULAR PRESSURES. A more marked decline occurs near the end of the tracing following a series of extrasystoles.

B. THE EFFECT OF ALTERNATELY ELEVATING RIGHT VENTRICULAR (RV) AND LEFT VENTRICULAR (LV) PRESSURES ON AORTIC PRESSURE (AO.) ARE CONTRASTED. Little change results from gradually elevating RV pressure, but a striking fall occurs when LV pressure is increased.

more than 5 beats per minute; in six it changed less than 5 beats per minute; and in two the changes were inconsistent. The specific chambers in which pressures were elevated did not appear to influence these results.

V. Effects of cessation of flow to the heart. In six experiments, when the inflow of blood to the heart was suddenly terminated, left ventricular pressure declined abruptly from an average value of 81/7 mm Hg, and reached levels of less than 8/5 mm Hg within several minutes after cessation of the heart beat. Right ventricular systolic pressure was less than 9 mm Hg prior to the cessation of perfusion and showed no significant change (Table II). In each dog systemic vascular resistance increased immediately, continued to rise for approximately 5 minutes and stabilized thereafter (Figure 6). These increases in systemic vascular resistance ranged from 14 to 51 per cent and averaged 25 per cent of the control values.

TABLE II

Hemodynamic effects of cessation of flow to the heart *

Dog no.	LV press.		LA press.		Ao press.		Increase Ao press.
	A	B	A	B	A	B	
	mm Hg		*cm H_2O*		*mm Hg*		%
1	85/6	6/0	8	6	65	98	51
5	75/9	1/0	8.5	0	70	82	17
6	83/7	1/0	10	6.5	64	73	14
8	87/6	8/5	10	7	88	113	28
11	83/7	1/0	10	6.5	94	105	12
13	75/7	0/0	7.5	3.5	90	115	29

* Columns A represent observations made just prior to cessation of flow to the heart. Columns B represent observations made following stabilization of all pressures several minutes following cessation of flow.

DISCUSSION

The innervated, hemodynamically isolated heart preparation employed in these experiments permitted precise study of the effects of altering intracardiac pressures on the separately perfused systemic circulation. Although the use of general anesthesia, an open chest and cardiopulmonary bypass inevitably compromised the reactivity of

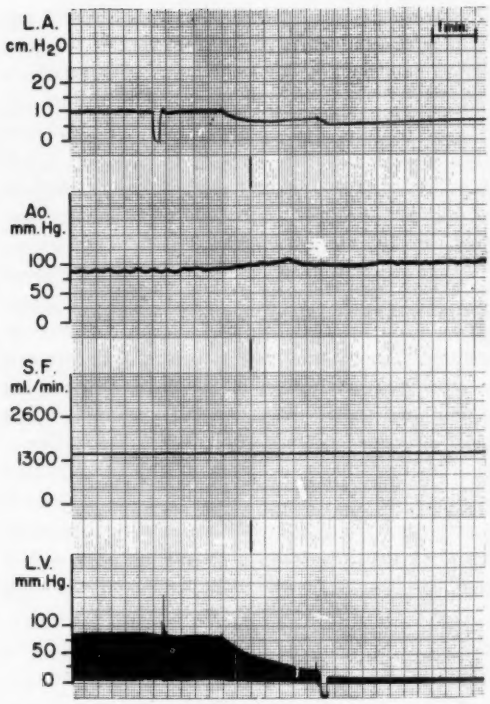

FIG. 6. DOG 8. EFFECTS ON AORTIC PRESSURE (AO.) OF CESSATION OF CARDIAC PERFUSION AND THE RESULTING DECLINE IN INTRACARDIAC PRESSURE. Cardiac perfusion was stopped at the instant that the left ventricular and left atrial pressures began to fall. An immediate elevation of aortic pressure is evident.

the preparation, these interventions were found necessary to investigate the nature of the peripheral circulatory changes following alteration of intracardiac pressures.

It was observed that a substantial decline in venous return resulted when pressures in the right and left ventricles were elevated. The significance of these changes in venous return is indicated by the large changes in the output of the extracorporeal pump which were necessary to obviate any alterations in intravascular blood volume. These changes in pump output provide a measurement of the decrease in cardiac output that would have occurred had the heart responded passively to the reflex change in venous return. In addition, when systemic perfusion was maintained constant, elevation of left heart pressures alone, or of both left and right heart pressures, resulted in an expansion of the intravascular blood volume. Increases in blood volume have also been recently reported by Salisbury, Cross, and Rieben following distention of the left ventricle with a balloon (8). Klussman, Van Citters and Rushmer, on the other hand, did not demonstrate any changes in limb circumference or liver diameter when suction devices were applied to portions of the cardiac walls (17).

The changes in venous return and systemic blood volume following elevations of intracardiac pressures reported herein resemble those that were observed when pressure within the isolated carotid sinuses were elevated or when the vasodilating drug, Arfonad, was administered (12). It was previously suggested that these alterations in venous return and systemic blood volume are most likely due to dilatation of the systemic venous bed (12), because of *1*) the large magnitude of the alterations in systemic vascular volume which occurred, and *2*) the fact that only 15 ml per kg of blood is estimated to be contained in the arterial and arteriolar vascular beds of the dog (18). Furthermore, arterial pressure varied inversely with total systemic blood volume, and it is therefore possible that the changes in the volume of the prearteriolar bed were actually opposite in direction to the alterations in total systemic blood volume. From the data presented above, it also appears likely that an inhibition of venous tone resulted from stimulation of the intracardiac baroreceptors. Since splenic volume is known to vary with alterations in sympathetic tone (19), it is of interest that the changes in blood volume resulting from elevating intracardiac pressures were also observed in the dogs that had been splenectomized previously.

Reflexes originating from the left side of the heart were first clearly demonstrated by Aviado and Schmidt (5). In the present experiments, when left ventricular systolic pressure was elevated but only minimal change in left ventricular diastolic and left atrial mean pressures occurred, systemic vascular resistance declined. These observations suggest the presence of left ventricular baroreceptors sensitive to changes in systolic pressure. Indeed, bursts of impulses during early systole have been recorded from cardiac afferent nerve fibers by several investigators (1, 2). However, since some enlargement of the left atrium probably accompanied minimal elevations

of left atrial pressure, the stimulation of left atrial receptors responsive to volume changes (20) cannot be completely excluded except in that experiment (Dog 16) in which isolated left ventricular perfusion resulted in absolutely no change in left atrial pressure. More striking alterations in vascular resistance were induced when left ventricular diastolic and left atrial pressures were elevated above 10 mm Hg (Figure 7). Since the hila of the lungs were ligated in all of the experiments carried out on Dog 2, the large pulmonary veins were distended together with the left atrium. Previous studies have suggested that the pulmonary veins (4, 10, 11, 21), as well as the left atrium (7, 20) contain baroreceptor endings, and it is likely that receptors in both of these areas participated in the observed reflexes.

Contrary to the observations of others (4, 11), only minimal declines in vascular resistance occurred when, in the absence of extrasystoles (Figure 5), pressures in the right side of the heart alone were elevated. However, in the present experiments, ligation of the hila prevented pressure elevation in the smaller pulmonary vessels, which perhaps contain receptors responsible for the decline in peripheral resistance observed by others when right heart pressures were elevated (11). In addition, in the present studies, right ventricular diastolic and atrial pressures were only moderately elevated and provided a less extreme stimulus than that utilized in previous investigations (4). Accordingly, it is suggested from the present experiments that the intracardiac receptors of greatest importance in the control of systemic vascular resistance are those sensitive to elevation of left ventricular diastolic pressure and/or left atrial pressure. It is possible that the ultimate stimulus for these receptors is the stretch that occurs when the volume of the cardiac chambers is increased. However, the possibility that baroreceptors in the coronary vessels are also of importance has not been excluded, since elevation

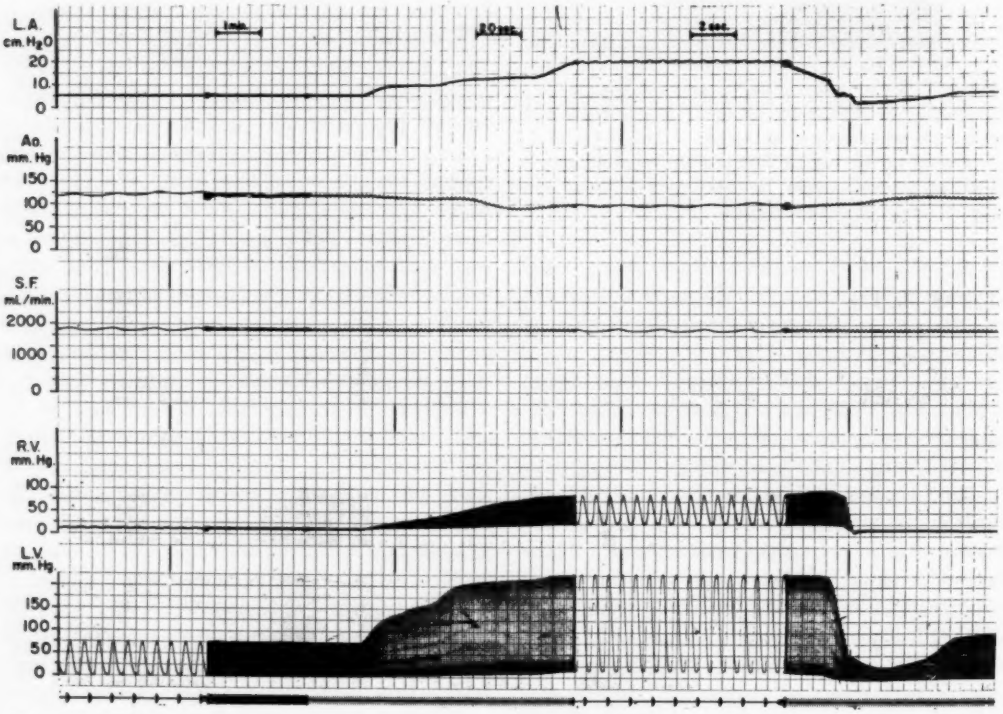

FIG. 7. DOG 2. TRACING SHOWING THE EFFECTS ON AORTIC PRESSURE OF STEP-WISE ELEVATION OF INTRACARDIAC PRESSURES. The most profound decline of aortic pressure occurred following elevation of mean left atrial (LA) pressure above 10 cm H_2O.

of left ventricular systolic pressure simultaneously raised the pressure in the coronary system.

It was observed that systemic vascular resistance fell when left ventricular systolic pressure was elevated in the range below the normal systolic blood pressure for the dog. This suggests that the left-sided intracardiac baroreceptors possess a low pressure threshold and, therefore, that these receptors may be continuously activated at normal intracardiac pressures. This possibility receives support from experiments in which rhythmic impulses, synchronous with both ventricular and atrial contractions, were recorded from cardiac afferent fibers (1, 2, 9). Furthermore, the initial rapid rise in arterial pressure (Figure 6) which occurred following cessation of cardiac perfusion suggests that a continuous inhibitory influence on vascular resistance originating from the heart had been removed; this observation provides additional evidence for tonic activity of the intracardiac receptors. The continued and progressive elevation in arterial pressure, following cessation of cardiac perfusion, however, may have resulted from stimulation by anoxia of receptors in the myocardium or coronary arteries.

In interpreting the action of reflexes originating from intracardiac receptors, the important effects of extrasystoles must be emphasized. It was observed in the course of these experiments that whenever extrasystoles occurred, whether during right- or left-sided pressure elevation, a striking decline in vascular resistance took place. This finding suggests that the intracardiac receptors, like those in the carotid sinus (*22*), are activated by rapid fluctuations in stimulation, regardless of the mean level of the stimulus applied. The occurrence of extrasystoles during elevations in right heart pressure thus make interpretation of subsequent reflex changes difficult, and may help to explain the results obtained by some previous investigators (4, 11).

The reflex effects of the intracardiac baroreceptors may be of importance in a variety of physiologic and pathologic circumstances. It would seem likely that they operate in conjunction with the sinoaortic mechanism in the regulation of both arteriolar and venous tone and therefore of arterial pressure and cardiac output. In addition, when intracardiac pressures alone are elevated, as in the presence of congestive heart failure or valvular stenosis, the resulting arteriolar and venous dilatation could serve to decrease the hemodynamic burden imposed on the heart both by diminishing systemic pressure and by decreasing venous return.

SUMMARY

The effects of alterations of intracardiac pressures on total venous return, system vascular volume and peripheral vascular resistance were studied in an experimental canine preparation. The systemic vascular bed was perfused, using an extracorporeal circulation, while pressures were independently varied in the innervated, but hemodynamically isolated heart.

Elevation of intracardiac pressure (either in all four chambers or only in the left side of the heart) in ten dogs resulted in an augmentation of systemic vascular volume which averaged 120 ml. In the three dogs in which the venous return was measured, it declined by an average of 25 per cent from the control levels. Significant decreases in systemic vascular resistance were observed when left ventricular systolic pressures were elevated, when left ventricular diastolic and mean left atrial pressures were also elevated, or when extrasystoles occurred. Only minimal alterations in vascular resistance were observed to accompany elevation of right heart pressures alone.

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THE EFFECT OF BUFFER LOADING UPON AMMONIUM EXCRETION IN THE DOG*

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According to present concepts, urinary ammonium and titratable acid represent, quantitatively, hydrogen ions eliminated from the body by the kidney (1). Although it is known that titratable acid excretion depends upon the amount of buffer in the urine and urine pH (2), and that ammonium excretion in man and dog is determined primarily by urine pH (3–6), it is not clear whether there is any fundamental interdependence of these two moieties of acid excretion. In previous studies where both were measured (7–10), variations in urine acidity could have affected either ammonium or titratable acid excretion predominantly and could thus have obscured a significant underlying relationship between the two.

In the present experiments in dogs, titratable acid excretion was increased progressively by intravenous sodium phosphate loading, and the effect on ammonium excretion was studied under conditions of relatively constant urine pH. Because of evidence that ammonium excretion is limited by rate of intracellular NH_3 production in acid but not in alkaline urine (4), experiments were carried out in both urine pH ranges. In one group of animals, ammonium excretion was stimulated by prior induction of metabolic acidosis. It was found that in each of these experimental situations, ammonium excretion was entirely independent of the buffer content of the urine and the rate of titratable acid excretion. Although these observations do not allow any conclusions concerning the intimate nature of ammonium transport into the urine, they suggest that the mechanism regulating ammonium excretion in the dog may be dissociated from total hydrogen ion excretion and those factors that determine the magnitude of Na^+–H^+ exchange. They also support the view that the availability of hydrogen ions does not ordinarily constitute a limiting factor in the accumulation of ammonium in the urine.

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METHODS

Fifteen experiments were carried out on 15 female mongrel dogs weighing between 10 and 22 kg. In 10 experiments, systemic acid-base balance was normal and the urine pH was adjusted to either an acid or a more alkaline range by infusion of sodium ferrocyanide or sodium chloride. In the other 5 experiments, ammonium excretion was augmented by inducing metabolic acidosis for 5 to 7 days prior to the acute experiment. Titratable acid excretion was increased progressively by infusion of sodium phosphate in concentrations calculated to deliver sodium at the same rate as during the preceding control periods. Anesthesia was induced by intravenous chloralose, 100 mg per kg, as a 1.5 per cent solution in 4 per cent dextrose. Additional small amounts were given as required.

Group I: Normal acid-base balance. Five animals were maintained on a salt-free diet for 7 to 10 days. After induction of anesthesia, a solution containing 30 mmoles per L of $Na_4Fe(CN)_6$ was administered at 4.5 ml per minute by means of a Bowman infusion pump. When a constant urine flow had been established, several timed urine collections were made. The solution was then replaced by one containing the following in millimoles per liter: Na_2HPO_4, 48; NaH_2PO_4, 12; $Na_4Fe(CN)_6$, 3. Fifteen to 45 minutes after starting this infusion, urine was collected for 5 additional periods.

The other 5 animals in this group were maintained on a normal diet. Sodium chloride, 135 mmoles per L, was infused and after a constant urine flow had been established, several urine specimens were collected. A sodium phosphate infusion was then begun and additional urine specimens obtained.

Group II: Chronic metabolic acidosis. Five dogs were fed 8 g of ammonium chloride daily for 5 to 7 days prior to the acute experiment. In order to assure a markedly acid urine, 0.1 N HCl in 4 per cent dextrose was administered for 30 to 60 minutes at the start of each experiment. Control urine specimens were collected during sodium ferrocyanide infusion. A solution containing 50 mmoles per L of Na_2HPO_4 and 25 mmoles per L of NaH_2PO_4 (pH 7.1) was then substituted for the ferrocyanide and additional urine collections were ob-

tained. Dextrose was added to each solution to adjust the final osmolarity to between 240 and 270.

Urine was collected through an indwelling catheter and the bladder emptied by manual compression at the end of each period. pH was determined on mid-point aliquots collected without exposure to air. Heparinized blood samples were collected from the femoral artery for determination of pH and CO_2 content.

Urine and blood pH were determined at 37° C without exposure to air, with a Cambridge model R pH meter. Plasma CO_2 content was determined manometrically by the method of Van Slyke and Neill (11). Urine ammonium was measured by the method of Folin and Bell (12) and inorganic phosphorus by that of Fiske and Subbarow (13). Sodium and potassium were determined by flame photometry with lithium as an internal standard. Chloride was measured by titration with silver nitrate, by a potentiometric method (14). Titratable acid was calculated from urine phosphate, urine pH and blood pH. (During phosphate loading, organic buffers contribute only a relatively small amount to total titratable acid excretion.) In this calculation, the pK'_2 of phosphoric acid was taken to be 6.8 in blood and in urine when urine pH was below 6.0. It has been shown that significant deviations in pK'_2 due to variations in urinary ionic strength may introduce appreciable errors in estimation of titratable acid when urine pH is above 6.0. In such urines, pK'_2 was calculated from an estimated ionic strength. An arbitrary addition of 0.025 was made to the ionic strength estimate to account for organic acids, bicarbonate, calcium, and other unmeasured ions. The theoretical considerations and methods of calculation of pK'_2 from ionic strength have been previously described (15).

RESULTS

Group I. The results of one of the experiments in this group are shown in detail in Table I. During the infusion of sodium ferrocyanide, urine pH varied between 5.04 and 5.30 and ammonium excretion between 27 and 30 μmoles per minute. With the subsequent administration of sodium phosphate, titratable acid excretion increased from 1 to 100 μEq per minute, and urine pH rose only about 0.3 unit. In spite of the marked increase in buffer content of the urine and titratable acid excretion, ammonium excretion was essentially unaltered. Blood pH and bicarbonate were in the low-normal range and did not change appreciably during the course of the experiment.

The results of the five experiments in which aciduria was induced by ferrocyanide infusion are shown in Figure 1 as solid circles. The circle marked by an asterisk represents the average ammonium and titratable acid values for all periods during sodium ferrocyanide administration. Urine pH values were between 4.8 and 5.8 in almost all instances and in individual experiments varied by no more than 0.6 unit. It is clear that increases in titratable acid excretion up to 133 μEq per minute had no discernible effect upon ammonium excretion. Sodium excretion remained essentially constant in three experiments, while in the other two there was a moderate fall when the ferrocyanide solution was replaced by phosphate. Blood pH and bicarbonate values were within normal limits during control periods and showed small increases during phosphate loading.

In Table II are shown the results of an experiment in which urine pH was more alkaline, owing to the administration of sodium chloride.

TABLE I

The effect of a sodium phosphate infusion upon ammonium excretion

		Urine								Plasma		
Time	Flow	Na	K	Cl	NH_4^+	P	pH	TA	TA+NH_4^+	pH	HCO_3	pCO_2
min	*ml/min*	*μmoles/min*	*μmoles/min*	*μmoles/min*	*μmoles/min*	*μmoles/min*		*μEq/min*	*μEq/min*		*mmoles/L*	*mm Hg*
0	Infusion: $Na_4FE(CN)_6$, 30 mmoles/L; dextrose, 110 mmoles/L at 4.5 ml/min											
65–85	1.5	261	78	4	29	2	5.16	1	30	7.31	20.7	42.5
85–100	1.7	346	87	4	28	2	5.24	1	29			
100–115	1.7	294	67	3	30	2	5.30	1	31	7.30	19.8	41.4
115–130	2.2	273	62	2	27	1	5.04	1	28			
130–145	3.1	357	69	5	30	1	5.29	1	31	7.31	21.7	44.4
147	Infusion: Na_2HPO_4, 48 mmoles/L; NaH_2PO_4, 12 mmoles/L; $Na_4Fe(CN)_6$, 3 mmoles/L; dextrose, 55 mmoles/L at 4.5 ml/min											
185–200	2.7	284	66	3	23	67	5.53	48	71			
200–215	2.6	263	63	1	24	83	5.54	60	84	7.34	22.7	43.4
215–230	2.2	214	56	2	22	91	5.52	66	88			
230–245	2.1	212	59	2	23	106	5.61	77	100			
245–260	2.3	223	59	2	26	137	5.57	100	126	7.36	22.8	41.5

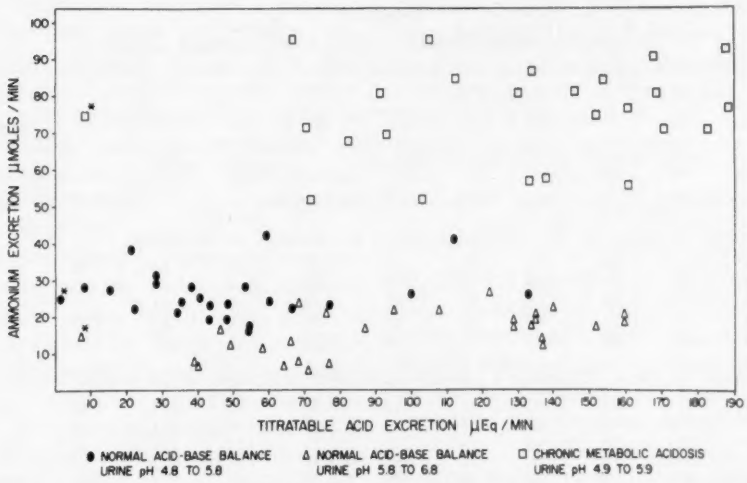
AMMONIUM EXCRETION μMOLES / MIN
TITRATABLE ACID EXCRETION μEq / MIN
● NORMAL ACID-BASE BALANCE URINE pH 4.8 TO 5.8
Δ NORMAL ACID-BASE BALANCE URINE pH 5.8 TO 6.8
□ CHRONIC METABOLIC ACIDOSIS URINE pH 4.9 TO 5.9

FIG. 1. THE EFFECT OF PROGRESSIVE INCREASES IN TITRATABLE ACID EXCRETION UPON THE CONCURRENT RATE OF AMMONIUM EXCRETION.

During the control periods, urine pH varied between 6.46 and 6.60 and ammonium excretion between 18 and 26 μmoles per minute. Titratable acid contributed only small amounts to total acid excretion. With the subsequent infusion of sodium phosphate, titratable acid excretion increased to 135 μEq per minute, while urine pH remained essentially constant. As can be seen from the table, the marked rise in titratable acid excretion was without effect on ammonium under these circumstances. Blood pH and bicarbonate rose moderately during phosphate loading.

The results of all five experiments in which this protocol was followed are shown as triangles in Figure 1. The average of ammonium and titratable acid values during sodium chloride administration is represented by the symbol marked with an asterisk. Urine pH values were between 5.8 and 6.8 in almost all periods and varied by no more than 0.5 unit in individual experiments. It is apparent from the figure that increases in titratable acid excretion up to 160 μEq per minute had virtually no effect upon the simultaneous excretion of ammonium. Sodium excretion rose in one experiment, fell in one, and remained essentially unchanged in the other three.

TABLE II

The effect of a sodium phosphate infusion upon ammonium excretion

		Urine									Plasma		
Time	Flow	Na	K	Cl	NH_4^+	P	pH	pK_2'	TA	TA+NH_4^+	pH	HCO_3	pCO_2
min	*ml/min*	*μmoles/min*	*μmoles/min*	*μmoles/min*	*μmoles/min*	*μmoles/min*			*μEq/min*	*μEq/min*		*mmoles/L*	*mm Hg*
0	Infusion: NaCl, 135 mmoles/L at 6.8 ml/min												
60–70	5.2	442	73	511	25	14	6.46	6.80	6	31	7.28	19.2	42.2
70–80	7.4	375	74	448	24	13	6.47	6.88	6	30			
80–90	7.0	434	77	496	26	14	6.46	6.84	7	33			
90–100	5.8	505	84	632	19	18	6.55	6.80	7	26	7.32	20.8	41.7
100–110	5.2	515	80	567	18	18	6.60	6.79	7	25			
112	Infusion: Na_2HPO_4, 60 mmoles/L; NaH_2PO_4, 15 mmoles/L; dextrose, 55 mmoles/L at 6.8 ml/min												
135–145	6.5	559	107	371	19	227	6.26	6.79	129	148	7.39	21.1	36.0
145–155	7.4	518	115	239	19	272	6.42	6.81	135	154			
155–165	8.0	624	116	254	18	315	6.55	6.80	134	152	7.36	22.8	41.5
165–175	7.2	691	122	266	18	353	6.64	6.78	129	147			
175–185	7.3	667	123	209	21	365	6.63	6.78	135	156	7.36	23.2	42.4

TABLE III

The effect of a sodium phosphate infusion upon ammonium excretion

	Urine									Plasma		
Time	Flow	Na	K	Cl	NH_4^+	P	pH	TA	TA+NH_4^+	pH	HCO_3	pCO_2
min	*ml/min*	*μmoles/min*	*μmoles/min*	*μmoles/min*	*μmoles/min*	*μmoles/min*		*μEq/min*	*μEq/min*		*mmoles/L*	*mm Hg*
0	Infusion: HCl, 0.1 N; dextrose, 220 mmoles/L at 6.8 ml/min									7.15	9.2	27.2
42	Infusion: $Na_4Fe(CN)_6$, 31 mmoles/L; dextrose, 72 mmoles/L at 6.8 ml/min											
60–70	10.4	342	88	124	76	20	5.52	12	88	7.02	6.2	25.0
70–80	10.3	349	82	118	71	17	5.87	9	80			
80–90	9.1	319	68	62	72	17	5.63	9	81			
90–100	6.7	415	74	60	72	16	5.36	9	81	7.01	6.9	28.0
100–110	4.5	481	89	51	76	19	5.20	11	87			
111	Infusion: Na_2HPO_4, 50 mmoles/L; NaH_2PO_4, 25 mmoles/L; dextrose, 28 mmoles/L at 6.8 ml/min											
135–145	7.1	374	92	46	81	142	5.48	91	172	7.14	6.9	20.8
145–155	7.4	370	85	48	81	196	5.39	130	211			
155–165	7.9	393	75	46	75	225	5.23	152	227	7.17	6.5	18.5
165–175	7.4	370	70	50	71	253	5.21	171	242			
175–185	6.8	374	68	48	71	274	5.27	183	254	7.16	6.9	19.9

As can be seen from the figure, ammonium excretion was generally higher in the animals with aciduria than in those with more alkaline urine.

Group II. In Table III is shown one of the experiments in which ammonium excretion was enhanced by prior induction of metabolic acidosis. Plasma bicarbonate was lowered further by infusion of 0.1 N HCl. During the administration of sodium ferrocyanide, urine pH varied between 5.20 and 5.87 and ammonium excretion between 71 and 76 μmoles per minute. The contribution of titratable acid to total acid excretion was small. As the result of sodium phosphate (pH 7.1) loading, titratable acid excretion rose to 183 μEq per minute, but it is apparent that this was without effect upon the concurrent excretion of ammonium. Urine pH remained within the narrow range of 5.21 to 5.48 and sodium excretion was essentially constant. Blood bicarbonate did not vary throughout the experiment, but pH rose and pCO_2 fell.

The results of the five experiments in this group are shown as squares in Figure 1. The symbol representing the average ammonium and titratable acid values during sodium ferrocyanide infusion is marked by an asterisk. Ammonium excretion was greater in this group than in Group I because of the stimulus of chronic metabolic acidosis. The wide spread in rates of ammonium excretion in the acidotic animals is not the result of differences in urine pH, since this varied by no more than 0.7 unit in individual experiments. It is, rather, related to differences in the duration of ammonium chloride ingestion. For example, the five points of lowest ammonium excretion are from the experiment in which ammonium chloride was fed for the shortest period of time. It is apparent, however, that increases in titratable acid up to 190 μEq per minute had no significant effect upon the rate of ammonium excretion. Sodium excretion remained essentially constant in all five experiments.

DISCUSSION

The present experimental observations demonstrate that, under conditions of relatively constant urine pH, progressive increases in titratable acid excretion can take place without having any significant effect upon the concurrent rate of ammonium excretion. This was found to be true in the presence of normal acid-base balance and also in chronic metabolic acidosis with markedly augmented rates of ammonium excretion. This dissociation of ammonium from titratable acid excretion is consistent with the view that NH_3 diffuses passively into an acid tubular fluid where it is "trapped" by conversion to NH_4^+ (4). The rate at which this physicochemical process proceeds depends primarily upon the pH gradient between tubule cell and lumen and should be independent of all changes in composition of the tubule fluid other than that of pH (4). It therefore follows that the addition of large amounts of buffer

to the tubular fluid and the associated acceleration of Na^+–H^+ exchange should not influence ammonium excretion. However, the results of these experiments are also compatible with the hypothesis that NH_4^+ gains access to the urine by an independent Na^+–NH_4^+ exchange mechanism (9, 16), and therefore do not provide critical information concerning the intimate nature of ammonium transport into the urine.

It has been demonstrated repeatedly that ammonium excretion in man and dog is inversely related to urine pH (1, 4, 5, 17), a fact which is most readily explained by the passive diffusion theory. According to the physical laws governing such a process, ammonium excretion should increase tenfold with each unit fall in urine pH if the tubular cell membrane is freely permeable to NH_3 and completely impermeable to NH_4^+ (4, 6). Stated in another way, there should be a linear relationship between urine pH and ammonium excretion when the latter is plotted logarithmically, and the regression coefficient defining the rate of change of log ammonium excretion per unit fall in urine pH should be -1.0 (6). The experimentally observed relationship in man and dog is indeed linear but the calculated regression coefficient is considerably less than unity (3, 4). In order to account for this discrepancy within the context of the diffusion hypothesis, it has been postulated that the rate of intracellular ammonia production limits ammonium excretion in acid urine and is insufficient for an equilibrium state to be achieved (4). This postulate is based on the observation that ammonium excretion varies with urine flow in the alkaline but not in the acid range of urine pH (4). As an alternate explanation, it seems reasonable that the availability or rate of diffusion of hydrogen ions into the tubular fluid might play a significant regulatory role in ammonium excretion, particularly in acid urine, since H^+ is necessary for the conversion of NH_3 to NH_4^+ prior to excretion. However, the present studies show that large increases in hydrogen ion excretion can take place in both alkaline and acid urine at a time when ammonium excretion is already maximal for the urine pH. This suggests that, in experimental situations where only endogenous amounts of buffer are present, hydrogen ions are available in excess and therefore do not constitute a limiting factor in ammonium excretion. Indeed, the present experimental observations indicate that the process regulating ammonium excretion in the dog may be largely independent of total hydrogen ion excretion and those factors that determine the magnitude of the Na^+–H^+ exchange mechanism.

SUMMARY

The relationship between ammonium and titratable acid excretion has been studied in dogs under conditions of relatively constant urine pH. It was found that progressive increases in titratable acid excretion due to sodium phosphate loading had no significant effect upon ammonium excretion in either normal or chronically acidotic animals. This dissociation of ammonium from titratable acid excretion is consistent with the view that ammonia accumulates in the urine by passive diffusion, since this process depends primarily upon the pH gradient between tubular cell and lumen and should be independent of all changes in composition of the tubular fluid other than that of pH. However, an independent Na^+–NH_4^+ exchange mechanism can also account for the present observations and, therefore, conclusions cannot be drawn from these experiments concerning the intimate nature of the tubular mechanism for ammonium excretion.

The finding that large increases in titratable acid excretion can take place without affecting urinary ammonium suggests that, in experimental situations where only endogenous amounts of buffer are present, hydrogen ions are available in excess and do not constitute a limiting factor in ammonium excretion. It appears, rather, that ammonium excretion in the dog may be largely independent of total hydrogen ion excretion and those factors that determine the magnitude of the Na^+–H^+ exchange mechanism.

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THE EFFECT OF HEMORRHAGE UPON ALDOSTERONE SECRETION IN NORMAL AND HYPOPHYSECTOMIZED DOGS*

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Acute hemorrhage has been demonstrated to stimulate aldosterone output in dog and man (1–3). Because of the relatively greater increment in aldosterone than in glucocorticoid secretion or excretion, a specific aldosterone-stimulating effect has been postulated. All of these studies, however, were performed in subjects with intact pituitaries. In view of the accumulating evidence (4–8) of the role of ACTH in regulating secretion of aldosterone, especially in the acute experiment, the role of the pituitary in the increment in aldosterone secretion following hemorrhage remains uncertain. The present experiments were designed to compare the effect of acute hemorrhage on aldosterone secretion in normal and in hypophysectomized dogs.

METHODS

Fifteen male mongrel dogs (9.5 to 15 kg) were studied. The normal group of 5 dogs had the right lumbo-adrenal vein cannulated by the technique of Hume and Nelson (9) on the day prior to the experiment. At the start of the experiment each dog was anesthetized with Nembutal and the right femoral vein and artery were cannulated, the latter for continuous blood pressure recording, using a Grass model 5 polygraph and a Statham strain gage. Ten- to 15-ml samples of adrenal vein blood were collected. Each sample was immediately replaced by dog bank blood to avoid the effects of intermittent small hemorrhages. After two to three control samples, collected at 20 to 30 minute intervals, 15 ml of blood per kg body weight was withdrawn from the femoral artery within 6 minutes. Collection of the first post-hemorrhage sample of adrenal vein blood was begun 10 minutes after the start of hemorrhage. An additional one or two samples were collected at 20- to 30-minute intervals. The shed blood was then transfused over 6 minutes and two to three post-transfusion samples were collected at intervals similar to those of the post-hemorrhage period. Following this last sample, 1 IU of ACTH (Upjohn) was administered through the femoral vein and collection of adrenal vein blood was begun 10 minutes later.

Ten dogs were hypophysectomized by the transbuccal route (10), and the right adrenal vein was cannulated on the experimental day. One hour after hypophysectomy, collection of adrenal vein samples was begun. The protocol thereafter was similar to that of the normal dogs. Blood samples were centrifuged immediately after collection, and the plasma was separated and frozen for subsequent analysis. Aldosterone and corticosterone were measured by the isotope derivative technique of Kliman and Peterson (11), and 17-dihydroxycorticoids by the Silber-Porter method (12). Secretion rates were calculated from the concentrations and the plasma flow per minute. Completeness of hypophysectomy was checked by gross examination of the hypothalamus and sella turcica.

RESULTS

Normal dogs. In Table I are represented the results from five normal dogs. In all five dogs following the acute hemorrhage there was an immediate increment in aldosterone secretion which persisted throughout the post-hemorrhage period. Following transfusion of the shed blood there was a decrement in aldosterone secretion to below post-hemorrhage levels in the first sample in four of the dogs. In all dogs, administration of ACTH resulted in an increment in aldosterone secretion which was greater than the preceding secretory rate, and in four dogs was even greater than the highest post-hemorrhage rate.

The changes in corticosterone and 17-hydroxycorticoid secretion showed two separate patterns. Three dogs (208, 214 and 215) with low control secretion rates showed significant rise in both corticosterone and 17-hydroxycorticoid secretions following hemorrhage, indicating an ACTH-effect.

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TABLE I

Effect of acute hemorrhage upon adrenocortical secretions in normal dogs

Dog no.	Control period			Post-hemorrhage period			Post-transfusion period			Post-ACTH period
	1	2	3	4	5	6	7	8	9	10
201										
Aldosterone*	56		16	54		36	25		3‖	81
Corticosterone†	1.1		1.4	1.2		1.0	1.1		0.07	1.6
17-OH corticoids†	11.8	14.7	12.6	11.4	10.6	9.9	9.7	0.7	1.3	14.1
Blood pressure‡	115/60		120/60	80/45		105/50	125/80		125/75	125/75
Plasma flow§	1.1	1.6	1.5	0.9	1.0	1.0	1.4	1.7	1.7	1.7
208										
Aldosterone*	14		9	47		24	16		4	37
Corticosterone†	0.14		0.18	1.8		1.4	0.71		0.17	1.8
17-OH corticoids†	7.0	0.4	1.2	10.8	6.3	9.2	4.6	5.3	0	10.9
Blood pressure‡	125/75		135/90	60/35		80/50	130/90		165/105	160/100
Plasma flow§	1.2	0.8	1.2	0.4	0.2	0.7	0.9	1.1	1.6	1.7
214										
Aldosterone*	0		7	18		48	63		30	98
Corticosterone†	0.73		0.15	0.37		0.90	0.97		0.39	3.0
17-OH corticoids†	0.2	1.0	1.5	3.4	6.0	5.7	5.5	1.3	2.6	16.1
Blood pressure‡	125/75		130/80	70/30		115/65	135/80		135/80	135/80
Plasma flow§	1.1	0.7	0.7	0.3	0.4	0.6	0.9	0.9	1.1	1.6
222										
Aldosterone*	11	13	8	36		37	24		10	69
Corticosterone†	1.3	1.6	1.3	1.8		2.1	1.9		1.4	1.2
17-OH corticoids†	7.9	8.0	6.5	7.6	7.9	8.0	7.5	7.8	6.9	8.5
Blood pressure‡	160/100		150/100	165/40		120/80	155/105		150/105	150/100
Plasma flow§	0.9	1.1	1.2	0.4	0.4	0.4	0.5	0.5	0.6	0.7
215										
Aldosterone*	10	4	2‖	25	14	7	2‖	4	6	33
Corticosterone†	0.78	0.52	0.13	1.1	0.61	0.77	0.49	1.2	1.3	1.5
17-OH corticoids†	6.4	3.4	0.3	4.1	4.3			9.1	9.6	7.9
Blood pressure‡	125/73		125/75	85/50		120/75	130/80		120/80	125/80
Plasma flow§	0.4	0.3	0.4	0.2	0.3	0.5	0.3	0.6	0.7	0.8

* Millimicrograms per minute.
† Micrograms per minute.
‡ Millimeters of mercury.
§ Milliliters per minute.
‖ The quantity of aldosterone actually measured was very low in these specimens.

Two dogs (201 and 222), with high control secretion rates, showed little change following hemorrhage. In two dogs (222 and 215), although aldosterone secretion decreased after transfusion, that of corticosterone and 17-hydroxycorticoid remained the same or increased. In contrast to the uniformly large increment in aldosterone secretion following ACTH, two of five dogs showed little or no rise in corticosterone and 17-hydroxycorticoid secretion. Another dog not represented in the table, in which only 17-hydroxycorticoids were measured, showed a similar result—namely, no increment in glucocorticoid secretion following ACTH. In each instance the pre-ACTH levels of corticosterone and 17-hydroxycorticoids were high. A lack of response suggested that the rates of synthesis and release of glucocorticoids were already maximal.

Hypophysectomized dogs. In Table II are shown the experimental data from the hypophysectomized dogs. Gross inspection of the hypothalamus and sella turcica revealed no evidence of pituitary tissue. Serial sections for histological examination were not routinely made. However, the low levels of glucocorticoid secretion are compatible with complete hypophysectomy. Acute hemorrhage caused a prompt rise of aldosterone secretion which was evident in the first post-hemorrhage period, in nine of ten hypophysectomized dogs. In six of the ten dogs, the rise

was sustained throughout the post-hemorrhage period. Transfusion of the shed blood resulted in a decrement in aldosterone in three of these six, although Dog 15–60 had an unexplained rise in aldosterone secretion in the last post-transfusion period. As in the normal dog, ACTH produced a marked increment in aldosterone secretion. The secretory level attained following ACTH was always greater than the level following hemorrhage. The control secretory rates of 17-hydroxycorticoids were low in all dogs. In eight of nine dogs there was no significant change in 17-hydroxycorticoid secretion following hemorrhage. One dog (62) showed a slight rise, but the change is probably within the errors of the chemical method and the levels were well below those of the post-hemorrhage normal dogs. Corticosterone secretion was low in the four dogs in which it was measured. Although there was a slight increment following hemorrhage, the values obtained were well below the values seen in the post-hemorrhage normal dog. Moreover, alterations in corticosterone secretion did not always parallel alterations in aldosterone secretion. Intravenous administration of ACTH resulted in a marked rise in both corticosterone and 17-hydroxycorticoid secretion in every dog to levels comparable with those in the post-ACTH period of normal

TABLE II

Effect of acute hemorrhage upon adrenocortical secretions in hypophysectomized dogs

Dog no.	Control period			Post-hemorrhage period			Post-transfusion period			Post-ACTH period
	1	2	3	4	5	6	7	8	9	10
46										
Aldosterone*	16		21	31	11	28	29		26	
17-OH corticoids†	0		0				0			
Blood pressure‡	125/90		125/90	100/75		120/90	130/105		100/70	
Plasma flow§	0.6		0.6	0.6	1.0	0.9	1.1		1.5	
51										
Aldosterone*	11		15	21	18	34	5‖	5‖	9	
17-OH corticoids†	0	0	0.9	0.9	0.6	1.0		0	0	
Blood pressure‡	100/80		120/70	80/40		100/50	130/75		125/70	
Plasma flow§	1.5		1.2	0.6	1.4	1.8	2.0	1.9	2.0	
53										
Aldosterone*	0		0	0		17	17		23	73
17-OH corticoids†	0	0	0							
Blood pressure‡	145/100		145/100	110/75		120/75	125/55		130/80	135/85
Plasma flow§	1.8	2.1	2.3	3.0	3.1	2.3	3.2	2.0	1.9	2.7
62										
Aldosterone*	6		0	22		8	9		23	97
17-OH corticoids†	0.1	0.1	0	1.3	1.3	1.1	0	0.1	0.9	3.6
Plasma flow§	1.2	1.4	1.5	1.3	1.5	1.4	1.4	1.3	1.2	1.4
55										
Aldosterone*	0		0	5		0	0		0	29
17-OH corticoids†	0	0.7	0							
Blood pressure‡	125/75		125/75	59/9		80/30	115/60		120/50	120/50
Plasma flow§	1.7	1.1	1.3	0.4	0.9	1.1	1.6	1.2	1.1	1.1
197										
Aldosterone*	8		8	26		31	21		20	51
17-OH corticoids†	0.7	1.2	0.7	0.6		1.8	1.7	0.7	1.2	6.5
Blood pressure‡	110/70		85/50	60/40		70/50	120/80		90/70	100/75
Plasma flow§	1.1	1.4	1.7	0.8		1.3	1.4	1.7	2.1	2.3

* Millimicrograms per minute.
† Micrograms per minute.
‡ Millimeters of mercury.
§ Milliliters per minute.
‖ The quantity of aldosterone actually measured was very low in these specimens.

TABLE II—(*Continued*)

Dog no.	Control period			Post-hemorrhage period			Post-transfusion period			Post-ACTH period
	1	2	3	4	5	6	7	8	9	10
204										
Aldosterone*	8		1‖	9		14	8		17	93
Corticosterone†	0.26		0.25	0.41		0.45	0.21		0.66	3.4
17-OH corticoids†	0	0.7	0	0	0	0	1.0	0	0	9.9
Blood pressure‡	135/85		135/80	85/50		125/90	125/100		130/100	125/100
Plasma flow§	1.8	1.9	1.8	1.0	0.8	1.2	1.1	0.9	1.1	1.4
301										
Aldosterone*	20	12	13	23	6	7	4	3	15	53
Corticosterone†	0.036	0.047	0.035	0.065	0.050	0.071	0.14	0.016	0.028	1.4
17-OH corticoids†	0.2	0	0	0.1		0		0.2	0	5.8
Blood pressure‡	150/100		140/85	60/30		100/60	125/75		125/80	125/80
Plasma flow§	1.9	1.6	1.4	0.9	0.9	1.1	1.2	1.1	1.0	1.2
3–60										
Aldosterone*	37	37	32	43		37	41	38	30	67
Corticosterone†	0.057	0.058	0.048	0.12		0.23	0.096	0.16	0.13	2.1
17-OH corticoids†	0	0	0	0		0.4	0.2	0.2	0.2	7.0
Blood pressure‡	175/105		160/100	65/30		100/50	160/95		120/70	125/75
Plasma flow§	1.0	1.1	0.9	0.7		0.4	0.8	1.0	1.0	1.0
15–60										
Aldosterone*	0		2‖	8	8.0	10	3.0	4	23	16
Corticosterone†	0.026	0.025	0.034	0.036	0.10	0.30	0.02	0.015	0.071	2.3
17-OH corticoids†	0	0	0	0	0	0	0	0	0	5.4
Blood pressure‡	150/105		150/105	140/105		150/105	175/110		170/120	170/120
Plasma flow§	1.4	1.8	1.7	1.5	2.1	1.2	0.8	0.4	1.6	2.6

dogs, indicating the presence of a responsive adrenal.

In Figure 1 is represented a summary of the aldosterone results. All values are the average of two or three samples in each period for each dog. Only eight of the hypophysectomized dogs received ACTH. The p values were obtained by the paired *t* test. The increment in aldosterone secretion following hemorrhage in the normal and hypophysectomized dogs is significant—$p < 0.1$. Following transfusion of shed blood there is a decrease in the secretory rates of normal dogs but no significant change in the hypophysectomized dogs. The highest level of secretion occurs post-ACTH with approximately the same levels in both groups. In the post-hemorrhage period, the mean secretory level of the normal dogs is significantly greater than that of the hypophysectomized dogs —$p < 0.05$. The secretory levels, however, do not differ significantly in the control, post-transfusion or post-ACTH periods between the two groups of dogs.

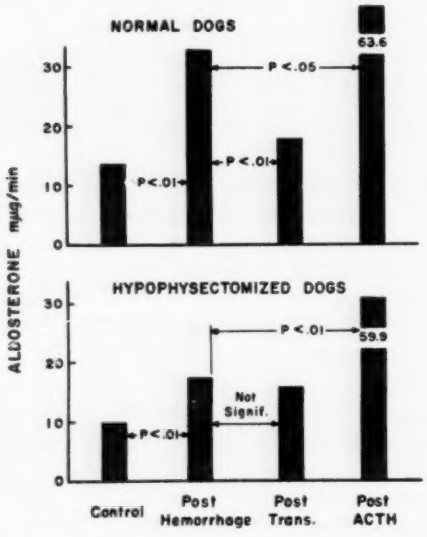

FIG. 1. SUMMARY OF ALDOSTERONE RESULTS IN NORMAL AND IN HYPOPHYSECTOMIZED DOGS.

DISCUSSION

Farrell, Rosnagle and Rauschkolb (1) were the first to show that hemorrhage stimulates aldosterone secretion in dogs. Although aldosterone was affected to a greater extent than cortisol, in several dogs the increment in cortisol secretion was appreciable. Fine, Meiselas

and Auerbach (2) noted an increment in the urinary excretion of aldosterone in normal humans following acute hemorrhage without any appreciable change in 17-hydroxycorticoid excretion. This increment lasted 1 to 2 days and could not be prevented by expansion of intravascular volume with the administration of 25 per cent albumin immediately after the hemorrhage. Bartter, Biglieri, Pronove and Delea (3) have also demonstrated that phlebotomy in sodium-depleted humans resulted in an increment in aldosterone excretion that was not prevented by administration of a volume of isotonic saline equivalent to the saline removed by phlebotomy. Spontaneous hemodilution and reinfusion of red blood cells did result in a decrement in aldosterone output. From their studies in the dog, Gann, Mills and Bartter (13) have concluded that the increment following hemorrhage is dependent upon an intact nerve to the thyrocarotid arterial junction and that the decrease in carotid arterial pressure specifically stimulates the release of aldosterone, presumably through release of a humoral factor. However, glucocorticoid secretion was never measured. Whether the nerve influences glucocorticoid secretion has not been determined. Experiments by Walker, Shoemaker, Kaalstad and Moore (14) have shown that acute hemorrhage may stimulate 17-hydroxycorticoid secretion.

The present study indicates that two mechanisms play a role in the increment in adrenocortical secretions following hemorrhage. The pituitary via ACTH release stimulates both glucocorticoid and aldosterone secretion. The marked increment in 17-hydroxycorticoid and corticosterone secretion following hemorrhage in the three normal dogs with low control secretory levels indicates an ACTH effect. Moreover, the demonstration that exogenous ACTH stimulated both glucocorticoid and aldosterone secretion in the normal and hypophysectomized dogs, and the greater increment in aldosterone secretion in the normal dogs following hemorrhage, are evidence that endogenous ACTH also can stimulate aldosterone output. The increment in glucocorticoid secretion following hemorrhage and the decrement following transfusion in normal dogs suggest that the release of ACTH is responsive to changes in intravascular volume, possibly mediated through the baroreceptor nerves.

A mechanism independent of the pituitary must also be involved, since aldosterone secretion rises following hemorrhage in the hypophysectomized dogs. A great deal of evidence indicates that this mechanism is humoral (15–18). The response within 15 minutes suggests that some component of intravascular volume, pressure or flow is the stimulus. The present experiments do not distinguish between a direct effect on the adrenal or stimulation by a humoral factor from a remote site. The studies of Farrell (15) indicate that such a site is in the head. However, Davis, Carpenter, Ayers and Bahn (19), who also found a rise in aldosterone secretion following hemorrhage in hypophysectomized dogs, present the rise in aldosterone secretion following hemorrhage in decapitated dogs as evidence for a more caudal origin of the proposed hormone.

The studies with ACTH in the normal dogs point out the hazards of comparing the relative change in glucocorticoid secretion with the relative change in aldosterone secretion, as an index of either ACTH or a specific aldosterone-stimulating factor. The metabolic state of the adrenal gland may alter the pattern of response to ACTH. Davis and colleagues (8) reported that infusion of ACTH into hypophysectomized, sodium-depleted dogs stimulated mainly aldosterone secretion. From the present study, it appears that in the acute experiment when glucocorticoids are being maximally produced, addition of ACTH stimulates mainly aldosterone secretion, as if it were a specific aldosterone-stimulating hormone. In Figure 2 is illustrated a possible explanation of this effect. Koritz and Peron (20) have proposed that the action of ACTH is twofold: to increase available TPNH by augmenting adrenal phosphorylase and to increase available steroid precursors. If at the maximal secretory rate of 17-hydroxycorticoids and corticosterone, the rate-limiting factor is available TPNH, then the steroid precursors could be synthesized to aldosterone through a less TPNH-dependent pathway than through corticosterone. Several workers (21, 22) have actually suggested another pathway directly from progesterone, although its dependence upon TPNH or other factors is not known.

When glucocorticoid synthesis is not at a maximal rate, ACTH stimulates both glucocorticoid and aldosterone. This is particularly evident in

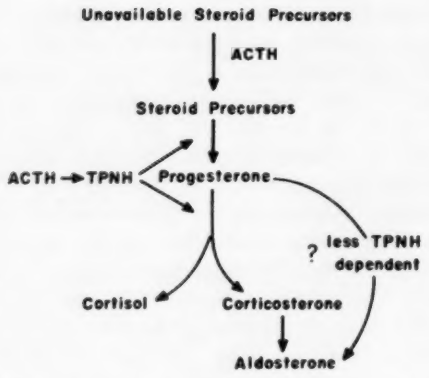

FIG. 2. SCHEMA OF ACTH STIMULATION OF ALDOSTERONE SECRETION.

the hypophysectomized dogs, in which ACTH always caused a marked increment in corticosterone and 17-hydroxycorticoid secretion. Unpublished observations by the authors, with successively increasing doses of ACTH in hypophysectomized or submaximally secreting dogs, show that the smallest dose affects glucocorticoid secretion first, while larger doses stimulate both glucocorticoid and aldosterone secretion.

These results cast some doubt on the validity of acute experiments performed in animals with intact pituitaries, since the adrenals of these animals are often secreting maximally. On the other hand, it should be kept in mind that the adrenal of the hypophysectomized dog may not respond as well to stimuli other than ACTH that increase aldosterone secretion, since ACTH is necessary to maintain steroidogenesis at a high level. This dependence appears to be clearly demonstrated in chronically hypophysectomized dogs, which do not respond to thoracic inferior vena cava constriction (8) as do normal dogs, and in patients with long-standing hypopituitarism who show a poor response to salt deprivation (23). However, whether this holds true to the same extent for the acutely hypophysectomized dog has not been clearly determined. Perhaps the failure to maintain the increased secretory level in the four hypophysectomized dogs is due to the absence of ACTH, although Farrell and associates (1) also noted that the secretory levels decreased despite continued blood loss in normal dogs. The present experiments, however, indicate that acute hemorrhage can stimulate the secretion of aldosterone independently of ACTH.

SUMMARY

The effect of an acute hemorrhage upon adrenocortical secretions has been studied in the normal and in the acutely hypophysectomized dog. Hemorrhage stimulated adrenocortical secretions by two mechanisms. In the normal dog, acute hemorrhage caused release of ACTH which stimulated both glucocorticoid and aldosterone secretion. However, if glucocorticoid secretion was maximal, only aldosterone secretion was stimulated, even by exogenous ACTH. Experiments in the hypophysectomized dog demonstrated that a mechanism independent of the pituitary stimulated primarily aldosterone secretion.

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ISOZYMES OF LACTIC DEHYDROGENASE IN HUMAN TISSUES*

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The heterogeneity of lactic dehydrogenase (LDH) activity in human serum and erythrocytes has been observed in several laboratories (1–7). If each of the electrophoretically-distinguishable enzymes in serum with LDH activity were derived from different tissues, in disease states involving a single organ the normal pattern of serum LDH activity would be altered according to the type of LDH released from the affected organ. This hypothesis was supported by analysis of sera from patients with myocardial infarction and leukemia (1–7). The results suggested that several tissues had characteristic electrophoretic distributions of lactic dehydrogenase activity. It was concluded that the patterns of serum LDH activity obtained by electrophoresis furnished more information regarding the site of pathology than did an examination of the LDH activity of whole serum (1–3). Furthermore, when several tissues were affected in a generalized disease process, as in hemorrhagic or endotoxic shock, it was found that all of the peaks of LDH activity in serum were elevated (8, 9), in contrast to the selective elevation of certain peaks in conditions involving individual tissues. The term isozyme was proposed by Markert and Møller to refer to the electrophoretically-distinguishable enzymes with similar substrate specificities (6). The present study demonstrates the heterogeneity of LDH activity in several human tissues and correlates the electrophoretic patterns of LDH activity obtained in tissues with those of sera in certain disease states.

MATERIALS AND METHODS

Four g of heart, of kidney, of liver and of skeletal muscle were obtained within 12 hours after the death of 8 patients from the following conditions: carcinoma of the cervix, bladder, breast, or kidney, brain tumor, diabetic gangrene of right foot, rheumatic heart disease and mitral stenosis, and rheumatic heart disease and aortic stenosis. No metastases to the liver, heart or kidney occurred in the cancer group. The tissues were washed thoroughly in cold normal saline to remove the majority of red cells and were rinsed in cold water to lyse any remaining erythrocytes. Homogenates were prepared in 10 ml of barbital buffer, pH 8.6, ionic strength 0.1, using a mortar and pestle and then submitting the tissues to further grinding in a Potter-Elvehjem motor-driven tissue homogenizer. Centrifugation of these extracts for 40 minutes at 15,000 G produced a supernatant fluid which was decanted and diluted with an equal volume of barbital buffer, pH 8.6. Four ml of the diluted supernatant was separated electrophoretically on a starch supporting medium in barbital buffer, pH 8.6, with an ionic strength of 0.1. After electrophoresis at 4° C for 36 hours at 400 v and 120 ma, each of the sections of the starch block was cut into 0.5 inch segments, the protein eluted with 5.0 ml barbital buffer and its concentration determined (10). An aliquot from each eluate was then assayed spectrophotometrically for LDH activity (11). Between 85 and 98 per cent of the LDH activity of the homogenate was recovered from the block following electrophoresis. Each eluate was incubated for 20 minutes at room temperature with 0.2 ml of 0.003 M diphosphopyridine nucleotide reduced (DPNH) and 2.5 ml of barbital buffer, pH 8.6, ionic strength 0.1. The mixture was transferred to a Beckman cuvet of 1 cm path length; 0.1 ml of 0.001 M sodium pyruvate was added, and the decrease in absorption at 340 mμ was measured in the Beckman DU spectrophotometer. Readings were obtained every 30 seconds for 3 minutes. One unit of dehydrogenase activity was defined as a decrease in the optical density at 340 mμ of 0.001 per minute.

In several experiments, a hemolysate prepared from human red cells (12) was fractionated electrophoretically and the eluates were assayed for LDH activity.

Human white cells were obtained from 40 ml of heparinized blood by the method described by Hirsch and Church (13). The LDH activity was extracted from this purified preparation of leukocytes by freezing in acetone and dry ice, followed by rapid thawing. The white blood cell extract was separated electrophoretically and the eluates were assayed for LDH activity.

Serum (5 ml each) from a patient with hepatitis and from a healthy control were separated simultaneously on a starch block.

The pH optimum for each peak of LDH activity was determined. An aliquot from the tube containing the

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maximal LDH activity in each peak was removed and added to barbital buffers, varying in pH from 7.4 to 9.2, all at 0.1 ionic strength. LDH activity was then reassayed as described above.

The Michaelis-Menten constants (Km) of lactic dehydrogenase were determined for each of the 5 peaks of LDH activity. One-thousandfold variations in the final concentration of sodium lactate (from 1.6×10^{-3} moles to 1.6 moles, pH 7.0, ionic strength 0.1) were employed. Diphosphopyridine nucleotide (DPN) was used (0.3 ml of a 0.05 M solution adjusted to pH 7.5) (14). Barbital buffer, pH 8.6, ionic strength 0.1, was added to an aliquot from the tubes with peak LDH activities. The final pH was 8.6 in a constant volume of 3.0 ml. The mixture was assayed for LDH activity and the Km calculated according to the method of Lineweaver and Burk (15).

RESULTS

Five electrophoretically-distinguishable peaks of LDH activity were observed in the homogenates of human tissues. According to the convention of earlier publications (2–4) enumeration will be made from the slower to the more rapidly migrating peaks. Figure 1 relates the mobility of these activity peaks to the electrophoretic pattern of serum proteins. Peaks 1 and 2 migrated with the mobility of γ-globulin, peak 3 with the mobility of β-globulin, peak 4 with the mobility of α_2-globulin and peak 5 with a mobility between α_1-globulin and albumin. Serum from a patient with hepatitis exhibited all five activity peaks; normally, peak 1 was present only in trace amounts and peak 2 contained less of the total activity, as shown by the control serum in Figure 1. Not all five activity peaks appeared in each of the tissues. Liver exhibited five peaks, whereas kidney, red cells and white cells revealed four, and heart and skeletal muscle had three peaks. The percentage of the total LDH activity found in each peak was relatively constant, as indicated by the values in Table I. Furthermore,

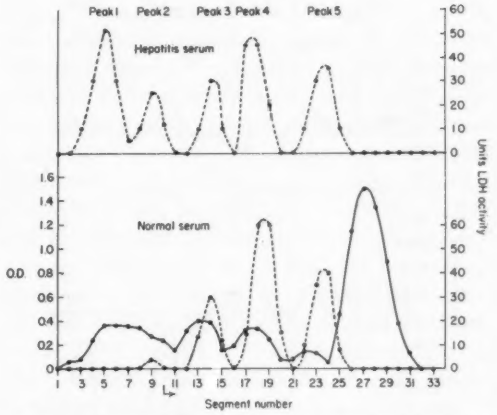

FIG. 1. DISTRIBUTION OF LACTIC DEHYDROGENASE ACTIVITY IN NORMAL HUMAN SERUM AND IN SERUM FROM A PATIENT WITH HEPATITIS. The lower curves show the electrophoretic pattern of normal human serum in the solid line and the distribution of lactic dehydrogenase activity of the same serum in broken lines. The upper curve shows the LDH activity in the serum from a patient with hepatitis separated on the same starch block. Note the appearance of peak 1 in this serum and the increased activity in peak 2, as compared with the normal serum below.

each tissue had a characteristic distribution of LDH activity. Most of the LDH activity in heart appeared in peak 5 with a smaller component in peak 4, whereas in liver the majority of the activity was found in peaks 1 to 3, and in skeletal muscle the largest portion of LDH activity was in peak 1. In kidney homogenate most of the LDH activity was divided between peaks 4 and 5, and in leukocyte extract peak 4 contained the majority of the LDH activity (Table I). The mobility of each of the activity peaks in one tissue resembled the mobility of the corresponding peaks in other tissues, as shown in Figure 2. Figure 3 also illustrates the similarity in mobility of activity

TABLE I

Lactic dehydrogenase activity in fractions of human tissues with standard deviations

Tissue	No. of cases	Per cent total activity				
		Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Heart	8			3 ± 2.5	24 ± 4.7	73 ± 5.6
Kidney	8		2 ± 2.9	11 ± 8.1	45 ± 5.2	42 ± 6.9
Liver	8	37 ± 9.6	24 ± 11.9	27 ± 7.2	8 ± 8.9	4 ± 6.6
Skeletal muscle	4	78 ± 8.95	17 ± 8.6	5 ± 4.2		
Hemolysate	5		1 ± 1.8	12 ± 2.9	44 ± 4.3	43 ± 2.2
White blood cells	5		6 ± 3.6	33 ± 6.1	49 ± 5.1	12 ± 3.6

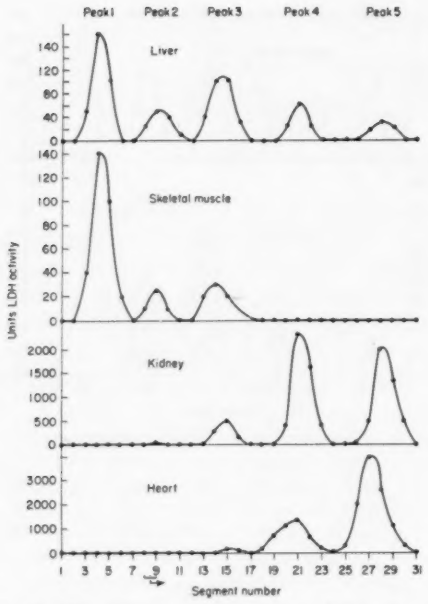

FIG. 2. DISTRIBUTION OF LACTIC DEHYDROGENASE ACTIVITY IN HOMOGENATES OF HUMAN HEART, LIVER, KIDNEY AND SKELETAL MUSCLE. These tissue homogenates were separated electrophoretically on the same starch block and are derived post mortem from a patient with carcinoma of the bladder.

peaks in one tissue to corresponding peaks in another tissue.

The pH optima of the 5 peaks of LDH activity were compared. Table II shows the close resemblance of these pH optima, which ranged from 7.8 to 8.5. The differences in pH optima among the five peaks of LDH activity were not statistically significant.

TABLE II

pH optima of five peaks of LDH activity

	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Heart			8.0	8.1	8.2
Hemolysate			8.0	8.2	8.5
Kidney			7.8	8.1	8.3
Liver	8.3	8.3			
Skeletal muscle	8.2	8.2			

TABLE III

Michaelis Menten constants (lactate) $\times$ 10^{-5} moles per L for 5 peaks of LDH activity

	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Heart			1.9	1.5	1.2
Kidney			1.0	2.0	1.0
Liver	4.4	4.8			
Skeletal muscle	5.6	4.2			

The Michaelis-Menten constants (Km) were calculated for the five activity peaks. The values obtained (Table III) revealed no significant differences among peaks 3, 4 and 5. The Km of peaks 1 and 2 (4.2 to 5.6 $\times$ 10^{-5} moles) were

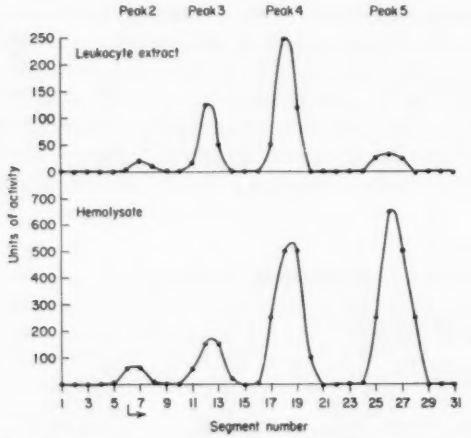

FIG. 3. DISTRIBUTION OF LACTIC DEHYDROGENASE ACTIVITY IN NORMAL HUMAN HEMOLYSATE AND LEUKOCYTE EXTRACT.

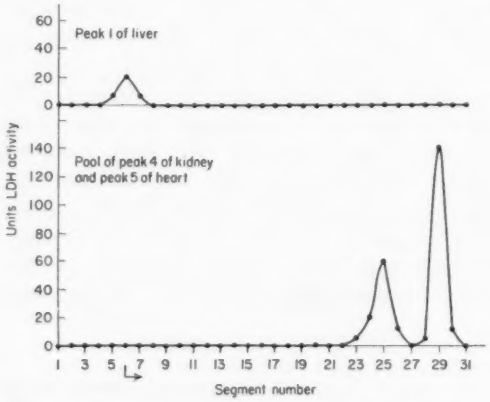

FIG. 4. THE PATTERNS OBTAINED WHEN PEAKS ISOLATED FROM A STARCH BLOCK WERE RERUN ON A SECOND BLOCK. The upper curve represents peak 1 of liver homogenate rerun on a second block. The lower curve represents a rerun of a pool of peak 4 from kidney homogenate and of peak 5 from heart homogenate on a second block. Note that when these peaks are rerun they do not alter in mobility or give rise to peaks not present in the initial run.

greater than the Km of peaks 3, 4 and 5 (1.0 to 2.0×10^{-5} moles).

Peak 5 from heart homogenate was pooled with peak 4 from kidney homogenate; the mixture was rerun on a second starch block under similar conditions, and the peaks had the same mobilities as in the starting material (Figure 4). Peak 1 from liver homogenate was rerun under identical conditions and no alterations in mobility from the starting material were observed (Figure 4).

DISCUSSION

Each of the homogenates of human tissue examined electrophoretically exhibited heterogeneity of LDH activity. Furthermore, each tissue had a constant number of activity peaks and a characteristic distribution of LDH activity in these peaks. The major portion of the LDH activity in heart homogenate was in peak 5 with a minor component in peak 4. Liver homogenate had the highest activity in peaks 1, 2 and 3, whereas most of the LDH activity of leukocytes resided in peak 4 with minor components in peaks 3 and 5. From analysis of alterations in the peaks of serum LDH activity in disease states, this type of distribution in tissues had been anticipated (1), from the hypothesis of enzyme release from damaged organs. In the sera of patients with myocardial infarction, a selective elevation of the LDH peaks with mobilities of α_1- and α_2-globulins was observed (1–3), and the present study reveals that these are the locations of LDH activity in heart homogenate. The report of an elevated peak of LDH activity in the α_2-globulin in certain leukemic sera (1) correlates with the observation that the majority of LDH activity in leukocytes is contained in this activity peak. The elevation of the LDH activity peak in the β-globulin and the appearance of a large activity peak in the γ-globulin in plasma from a patient with hepatitis are in harmony with the observation that the majority of LDH activity in liver homogenate resides in peaks with similar mobilities. Analagous to the appearance of a large LDH activity peak in serum during hepatitis is the appearance of additional peaks of leucine aminopeptidase activity in serum during liver disease (16). Both observations provide further support for the hypothesis that elevations in serum enzyme activity during several disease states are attributable in part to the liberation of enzymes from damaged organs.

Data of Hess (5) and Hill (17) are in accord with the reports of alterations in activity peaks of serum in cases of myocardial infarction and leukemia. However, Hill, employing curtain electrophoresis and a different method of obtaining leukocytes (17), localized the majority of LDH activity in leukocytes and erythrocytes in peak 3, whereas our data indicate that the majority of leukocyte LDH activity is contained in peak 4 and that peaks 4 and 5 contain most of the erythrocyte LDH activity. Wieme, utilizing agar gel electrophoresis, revealed 5 peaks of LDH activity in human serum (7). Peaks 1 and 2 were not constantly present and combined represented approximately 10 per cent of the total activity, the majority of the activity being distributed among peaks 3, 4 and 5. Wieme found that in patients with hepatitis, peaks 1 and 2 contained most of the serum LDH activity (7). This observation is confirmed in the present paper.

There are many examples of different molecular forms of enzymes in one organism and even in one tissue. Wieland and Pfleiderer observed several electrophoretically-distinguishable enzymes with LDH activity in several organs of the rat (18). The heterogeneity of pepsin (19), chymotrypsin (20), cytochrome C (21), ribonuclease (22), lysozyme (23), enolase (24), alkaline phosphatase (25), leucine aminopeptidase (16), ceruloplasmin (26), and cholinesterase (27) has been demonstrated. Emphasizing the similarities in substrate specificities of several enzymes previously shown to be heterogeneous, Markert and Møller proposed the useful term *isozyme* (6). They clearly distinguished isozymes, which exhibit similar substrate specificities and which usually resemble one another in several additional physicochemical characteristics, from different enzymes with broad substrate specificities which may overlap. Since comparison of the physicochemical factors in a group of electrophoretically-distinguishable enzymes which react with a common substrate is often difficult, caution must be exercised to avoid calling different enzymes with overlapping substrate specificities isozymes.

It appears unlikely that the differences in electrophoretic mobility of the LDH activity peaks are attributable to aggregation. Patterns similar

to those obtained on a starch block, which separates proteins primarily by charge, are obtained on a starch gel (7), which separates protein according to molecular weight and shape as well as according to charge. Further evidence against aggregation is the monodispersion of whole serum LDH activity in the ultracentrifuge (28).

Studies of pH optima and Km are usually sufficient to distinguish between the active centers of different enzymes. The differences in Km of peaks 1 and 2 from peaks 3, 4 and 5 suggest that the active centers of peaks 1 and 2 differ from those of peaks 3, 4 and 5. It is anticipated that further differences between each of the 5 isozymes will be established in the future. Previous studies showed that the inhibition behavior of peaks 3, 4 and 5 of hemolysate was identical (2). The possibility arises that the active centers engaged in catalyzing the pyruvate-lactate reaction in the five LDH isozymes are similar. The variations in electrophoretic mobility of the five LDH isozymes may be explained by postulating structural differences in the enzyme other than at the active center. These differences might determine the location of an enzyme within the cell (29) and could function in binding the enzyme to a particular organelle within a specialized cell. It is conceivable that one gene controls the catalytic site and that other genes or the chemical environment of the cell determine the attachment of the enzyme (29). This hypothesis may be applicable to the species differences in insulin, ACTH and ribonuclease (30). Recent studies of polymorphism in pseudocholinesterase reveal that the mutant enzyme differs from the normal type in pH optima and in affinity for different substrates, and that the mutant enzyme fails to react with acetylcholine (31). Here, the gene probably determines the polypeptide chain of the catalytic active center of the enzyme.

During the preparation of this manuscript, two extensive studies were published on fractionation of LDH activity in mammalian tissues employing starch gel electrophoresis (32, 33). Although the gel technique yielded results similar to those of the present study, detailed quantitative comparison of the results is rendered difficult by trailing of LDH activity on the starch gel in some of the experiments (32).

SUMMARY

1. Heterogeneity of lactic dehydrogenase (LDH) activity in the electrophoretically separated homogenates of human heart, kidney, liver, skeletal muscle, and extracts of leukocytes and erythrocytes is described. Five peaks of LDH activity were found in liver; four in kidney, leukocytes and erythrocytes, and three in heart and skeletal muscle. Each tissue exhibited a characteristic distribution of LDH activity: liver and skeletal muscle had the greatest activity in peaks 1, 2 and 3; whereas heart, hemolysate and kidney had most activity in peaks 4 and 5. Leukocytes and serum showed highest activity in peak 3.

2. The electrophoretic patterns of LDH activity in human tissues correlated with alterations in serum activity in disease states. In the serum from a case of hepatitis, a large peak of LDH activity is described which is present only in trace amounts in normal serum. This activity peak had the same mobility as the peak in liver with greatest LDH activity.

3. These observations suggest that assay of the LDH activity in the fractions of electrophoretically separated serum permits a greater specificity in localizing pathology than does assay of whole serum LDH activity.

4. Studies of Michaelis-Menten constants of the five peaks of LDH activity show peaks 1 and 2 to differ from peaks 3, 4 and 5. The possibility that the active centers of peaks 1 and 2 are different from the active centers of peaks 3, 4 and 5 is discussed.

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THE EFFECT OF HYPERCAPNIA ON ESTIMATED HEPATIC BLOOD FLOW, CIRCULATING SPLANCHNIC BLOOD VOLUME, AND HEPATIC SULFOBROMOPHTHALEIN CLEARANCE DURING GENERAL ANESTHESIA IN MAN *

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Splanchnic circulatory adjustments during general anesthesia in man are difficult to assess in the absence of precise information regarding the depth of anesthesia and the regulation of gas exchange. The vasoconstriction responsible for the fall in hepatic blood flow that has been reported by several investigators (1, 2) may be attributable to the anesthetic agents themselves, to changes in venous return following reduction in activity and tone of skeletal muscles (3), to hypoxia or to hypercapnia. Of all of these the last has been particularly difficult to detect and control (4). Now, however, the availability of satisfactory muscle relaxants (5) and the development of mechanical devices for the maintenance of a suitable respiratory pattern (6) have made it possible to achieve constancy in anesthetic level, oxygenation, and carbon dioxide elimination. Comparable "steady state" measurements of splanchnic hemodynamic parameters can be made before and during hypercapnia, because ventilatory excursion and rate can be fixed by artificial respiration after the administration of neuromuscular blocking agents, and the arterial carbon dioxide content can be altered as desired by appropriate changes in the breathing mixture. Under these circumstances, the volume of blood held within the splanchnic vasculature is determined only by local venomotor activity and by the balance between arteriolar resistances. Interference by variations in intra-abdominal pressure that might otherwise arise from excessive and unpredictable movements of the diaphragm are eliminated. Data of value in elucidating the vascular response to hypercapnia and anesthesia may therefore be obtained from measurements of splanchnic blood volume as well as blood flow (7). In the study reported in this paper, mechanically controlled light anesthesia (thiopental-nitrous oxide) alone appeared to have no effect upon the splanchnic bed, suggesting that extraneous factors may have been influential in producing the changes previously ascribed to anesthesia. In contrast, hypercapnia was associated with splanchnic vasoconstriction and with a reduction in splanchnic blood volume that was consistent with preponderant mesenteric and hepatic arteriolar constriction, splanchnic venoconstriction, or both.

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METHODS

The subjects for this study were 19 patients (9 male and 10 female, between 21 and 60 years of age) from the Surgical Service of the Presbyterian Hospital. None presented clinical evidence of cardiovascular, renal, hepatic or biliary tract disease. Each was examined in the fasting state immediately prior to a scheduled operative procedure of relatively short duration such as inguinal herniorrhaphy, excision of pilonidal cyst or biopsy of thyroid nodule. The effects of the preanesthetic medication alone were evaluated in 6. The effects of anesthesia and hypercapnia were studied in 13 subjects who had been given 8 to 15 mg of morphine sulfate and 0.4 to 0.5 mg of scopolamine hydrobromide intravenously, depending upon body weight, approximately 1 hour prior to hepatic venous catheterization.

Following control determinations, light anesthesia was induced by 150 to 400 mg of thiopental sodium given intravenously in divided doses and maintained by the administration of a mixture of 70 per cent nitrous oxide and 30 per cent oxygen, using an endotracheal tube without rebreathing. A 1.0 per cent solution of succinyl-

choline chloride in 5 per cent dextrose in water was infused intravenously at a rate sufficient to maintain apnea and immobility, permitting mechanical control of respiration.

Ventilation of the apneic patient was controlled by a "servorespirator" in which end-expiratory carbon dioxide tensions (P_{ACO_2}) (Liston-Becker infrared analyzer) automatically regulated the inflating pressure (6). This device is capable of maintaining the arterial carbon dioxide tension (Pa_{CO_2}) within ± 3 mm Hg for hours under the conditions employed in this study. After a period of 10 to 20 minutes for stabilization of P_{ACO_2} at approximately 40 mm Hg, all measurements (except total blood volume) were repeated. The P_{ACO_2} was determined with each breath and the Pa_{CO_2} was determined at 9- to 36-minute intervals during multiple sampling periods.

After completion of sampling, the respirator was reset to maintain a P_{ACO_2} of 60 mm Hg. This was accomplished in 4 subjects by reduction of the inspiratory pressures and in 9 subjects by the administration of a mixture of 3 per cent carbon dioxide, 70 per cent nitrous oxide and 27 per cent oxygen at essentially unchanged inspiratory pressures. Approximately 10 minutes was required to attain a new stable carbon dioxide tension. All measurements were repeated after 5 to 15 minutes at the altered P_{ACO_2}. In 4 subjects the period prior to anesthesia was omitted and the comparison made only between an initial period of eucapnia and a subsequent period of hypercapnia during anesthesia, while in 3 additional subjects the sequence was reversed.

Hepatic blood flow (EHBF) was estimated by the sulfobromophthalein (BSP) method (8), total blood volume by radioactive iodinated serum albumin dilution, circulating splanchnic blood volume (SBV) by the regional dilution method (9), and arterial pressure by auscultation. Mean arterial blood pressure (MBP) was

TABLE I

Effect of morphine and scopolamine given intravenously on splanchnic hemodynamics and sulfobromophthalein removal *

Subject	Period	Dose	BSP	EHBF	MBP	SVR	SBV	E%	C_{BSP}
		mg	*mg%*	*ml/min*	*mm Hg*	*units*	*ml*		*ml/min*
A	Control		0.94	1,060	92	0.087	490	55.6	350
	Scopol.	0.5	0.92	950	96	0.101	570	62.4	360
	Morph.	10.0	1.01	840	94	0.112	480	62.8	320
B	Control		1.03	1,120	92	0.082	750	53.9	350
	Scopol.	0.5	0.99	1,200	94	0.078	650	52.5	360
	Morph.	7.0	1.01	1,220	82	0.067	730	47.6	340
C	Control		0.79	1,080	93	0.086	910	59.6	380
	Scopol.	0.5	0.97	1,050	92	0.088	1,200	49.9	310
	Morph.	10.0	1.08	910	89	0.098	830	52.9	280
D	Control		0.68	1,560	89	0.057	1,110	56.8	460
	Scopol.	0.5							
	and morph.	10.0	0.71	1,540	92	0.060	1,040	51.7	420
E	Control		0.95	1,030	85	0.083	710	61.0	370
	Scopol.	0.5							
	and morph.	10.0	0.97	1,040	86	0.083	750	62.7	370
F	Control		0.67	1,620	83	0.051	1,130	53.8	510
	Scopol.	0.5							
	and morph.	10.0	0.71	1,350	84	0.062	950	57.7	460
Mean	Control		0.843	1,245	89	0.074	850	56.8	403
± SD			0.370	269	4.2	0.015	250	2.7	66
Mean	Scopol.		0.914	1,150	88	0.080	797	55.9	365
± SD	and morph.		0.399	270	4.6	0.022	196	6.1	66
Mean Δ			+0.072	−95	−1.2	+0.006	−53	−0.9	−38
± SD Δ			0.277	147	4.9	0.013	76	5.9	35
t			0.64	1.6	0.6	1.1	1.7	0.37	2.62
p			>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	<0.05

* After control measurements, Subjects A, B, and C received scopolamine and measurements were repeated within 26 minutes. Morphine was then administered and measurements completed within 24 minutes. Subjects D, E, and F received morphine and scopolamine together and measurements were completed within 25 minutes. Abbreviations: BSP, plasma sulfobromophthalein concentration; EHBF, estimated hepatic blood flow; MBP, mean arterial blood pressure; SVR, splanchnic vascular resistance; SBV, splanchnic blood volume; E%, sulfobromophthalein extraction percentage; C_{BSP}, sulfobromophthalein plasma clearance; SD, standard deviation.

estimated to be the sum of diastolic plus one-third of the pulse pressure. Hepatic BSP clearance (C_{BSP}) was calculated as the BSP removal rate divided by the prevailing plasma concentration and was expressed as milliliters of plasma cleared of BSP per minute. The splanchnic vascular resistance (SVR) was expressed in arbitrary units as the mean arterial blood pressure divided by the hepatic blood flow. Possible changes in the central venous pressure have been neglected in this calculation.

Assiduous attempts were made to maintain a steady state of anesthesia and arterial CO_2 tension. Measurements of hemodynamic variables were discarded when the simultaneous analysis of end-expiratory gas revealed fluctuation of the P_{ACO_2}. In addition, changes in the arterial BSP concentration greater than 0.020 mg per 100 ml per minute were regarded as invalidating a measurement of flow. All values presented in the tables are averages of two or more determinations except in Subjects 1 and 3, in whom only one valid determination of EHBF could be obtained during hypercapnia.

The arterial blood pH was determined anaerobically at 37° C in a modified Cambridge glass electrode pH meter (10) within 1 minute of sampling. Arterial plasma CO_2 content, or in some cases the whole blood CO_2 content, was determined in the Kopp-Natelson microgasometer (11). The Pa_{CO_2} was calculated from the nomogram of Singer and Hastings (12). Arterial oxygen saturations were determined in the microgasometer or by the spectrophotometric method of Nahas (13). The effect of BSP on the Nahas method was investigated and found to be negligible in the concentrations employed (1 to 2 mg per 100 ml).

RESULTS

Effects of scopolamine and morphine (Table I)

Scopolamine alone (Subjects A, B, C) produced no consistent changes. Morphine given after (Subjects A, B, C) or simultaneously with scopolamine (Subjects D, E, F) produced reductions of less than 10 per cent in EHBF and C_{BSP}. Smythe and Gilmore (14), using the same methods, have also reported the absence of an effect of morphine upon the splanchnic circulation in dogs. The mean values for the various splanchnic circulatory parameters (Table I) did not change significantly after administration of scopolamine, with or without morphine. It was concluded, therefore, that the use of premedication would not significantly alter the effects of anesthesia

TABLE II

Effect of nitrous oxide-succinylcholine anesthesia without hypercapnia on splanchnic hemodynamics and sulfobromophthalein removal *

Subject	Period	Pa_{CO_2}	BSP	EHBF	MBP	SVR	SBV	E%	C_{BSP}
		mm Hg	*mg%*	*ml/min*	*mm Hg*	*units*	*ml*		*ml/min*
1	Control	42	1.11	1,070	93	0.087	770	61.7	310
	Anesth.	33	1.39	1,340	91	0.068	1,210	37.1	230
2	Control		0.65	1,640	95	0.058		71.8	660
	Anesth.		0.72	1,640	120	0.073		67.9	600
3	Control	29	1.59	1,000	93	0.093	940	43.1	240
	Anesth.	44	2.28	780	93	0.119	590	36.4	160
4	Control	36	0.91	1,440	93	0.065	1,320	59.5	410
	Anesth.	38	0.63	2,110	103	0.049	1,260	57.5	590
5	Control	39	0.86	1,280	85	0.067	1,150	72.3	480
	Anesth.	41	1.03	1,070	91	0.085	930	70.7	390
6	Control		1.30	820	85	0.104	700	62.1	290
	Anesth.		1.53	1,170	122	0.104	950	44.5	260
Mean	Control	37	1.07	1,208	90.7	0.079	976	61.8	398
± SD		5.6	0.34	303	4.4	0.018	248	10.5	155
Mean	Anesth.	39	1.26	1,351	103.3	0.083	988	52.4	372
± SD		4.6	0.61	469	14.4	0.025	268	15.2	188
Mean Δ		2.5	0.193	143	12.7	0.004	12	—9.4	—26.7
± SD Δ		9.8	0.316	350	15.4	0.019	328	9.6	103.4
t		0.51	1.5	1.0	2.0	0.53	0.08	2.4	0.63
p <		0.7	0.2	0.4	0.1	0.7	0.9	0.1	0.6

* Abbreviations: Pa_{CO_2}, arterial carbon dioxide tension; otherwise as in Table I.

TABLE III

Effect of hypercapnia on splanchnic hemodynamics and sulfobromophthalein removal during nitrous oxide-succinylcholine anesthesia *

Subject	Period	Pa_{CO_2}	BSP	EHBF	MBP	SVR	SBV	E%	C_{BSP}
		mm Hg	*mg%*	*mg/min*	*mm Hg*	*units*	*ml*		*ml/min*
1	E	33	1.39	1,340	91	0.068	1,210	37.1	230
	H	61	1.74	1,200	105	0.088	920	31.2	180
2	E		0.72	1,640	120	0.073		67.9	600
	H		1.17	1,290	107	0.083		53.8	370
3	E	44	2.28	780	93	0.119	590	36.4	160
	H	56	3.31	630	130	0.206	750	30.2	100
4	E	38	0.63	2,110	103	0.049	1,260	57.5	590
	H	50	0.73	1,540	106	0.069	890	43.7	320
5	E	41	1.03	1,070	91	0.085		70.7	390
	H	59	0.96	1,140	120	0.105		56.0	330
6	E	32	1.53	1,170	122	0.104		38.3	250
	H	57	1.54	800	112	0.140		39.6	170
7	E	36	3.13	520	84	0.162	910	40.0	120
	H	61	2.85	550	97	0.176	470	29.1	90
8	E	38	1.43	1,430	98	0.069	560	24.2	220
	H	54	1.36	1,350	96	0.071	600	19.5	170
9	E	36	1.75	470	80	0.170	1,100	65.2	170
	H	51	2.11	490	83	0.167	920	43.1	110
10	E	39	1.14	1,710	77	0.045	1,530	29.6	270
	H	54	0.87	1.030	92	0.089	1,130	50.3	270
11†	E	40	0.83	980	101	0.103	12,90	80.7	450
	H	54	0.70	890	97	0.109	760	78.7	400
12†	E	44	1.09	1,120	80	0.071	660	55.4	350
	H	56	0.87	1,330	102	0.077	740	50.6	370
13†	E	39	2.38	590	89	0.151	570	43.1	150
	H	63	2.24	450	81	0.180	540	63.7	160
Mean	E	38.3	1.486	1,148	94.5	0.098	968	49.7	304
± SD		3.8	0.404	515	14.8	0.043	357	18.4	168
Mean	H	56.3	1.572	976	102.2	0.120	772	45.3	234
± SD		4.2	0.872	382	14.1	0.050	201	16.6	118
Mean Δ		18.0	0.086	—172	—7.6	0.0231	—196	—4.4	—70
± SD		5.9	0.389	256	16.2	0.024	244	13.3	89
t		10.7	0.80	2.42	1.7	3.36	2.54	1.2	2.83
p <		0.001	0.5	0.05	0.2	0.01	0.05	0.3	0.02

* Abbreviations: E, eucapnia; H, hypercapnia. Other abbreviations as in Tables I and II.
† The period of hypercapnia preceded the period of eucapnia in these subjects.

and hypercapnia under the conditions of these studies.

Effect of anesthesia and hypercapnia (Tables II and III)

The arterial carbon dioxide tension was not changed by anesthesia alone in four subjects (Table II). The mean Pa_{CO_2} in 12 subjects during eucapnia (E, Table III) was 38 mm Hg, whereas that during hypercapnia was 56 mm Hg, a change ($p < 0.001$) of + 47 per cent. The relatively constant deviations from the expected values of 40 and 60 mm Hg were largely explicable on the basis of systematic errors in infrared CO_2 monitoring attributable to nitrous oxide (15).

Arterial oxygen saturation during anesthesia

was maintained at or above control values in all cases, with the exception of Subject 5 in whom values of 91 and 89 per cent were obtained by the Nahas method. Subsequently this patient was found to have an arterial oxygen saturation of 93 per cent at rest, as determined by Van Slyke analysis. The data obtained were consistent with those of the 12 other subjects and were included in the final summation.

Mean arterial blood pressure increased 13 mm Hg on the average in 6 subjects (Table II) following the induction of anesthesia, and increased 8 mm Hg on the average in 13 anesthetized subjects (Table III) folowing the elevation of Pa_{CO_2}. Striking rises occurred in isolated instances. However, neither average change was statistically significant.

Estimated hepatic blood flow and the splanchnic vascular resistance did not change significantly with the induction of anesthesia. The estimated hepatic blood flow fell in 9 of the 13 subjects when the arterial P_{CO_2} rose. The mean fall in all 13 was 178 ml per mniute, or 15.4 per cent, a probably significant reduction ($p < 0.05$). The MBP increased in each subject in whom EHBF rose during hypercapnia. For the entire group, SVR in arbitrary units rose 22.4 per cent ($p < 0.01$). This value increased in 8 subjects and was unchanged (less than 10 per cent variation) in 5. It fell in none.

Splanchnic blood volume did not change significantly on the average with anesthesia prior to hypercapnia. Individual changes were erratic in direction and extent. With elevation of Pa_{CO_2} the mean SBV fell from 983 to 772 ml, a change of 211 ml or 21.4 per cent ($p < 0.05$). The SBV fell in 6 patients, rose in 1, and was unchanged (less than 10 per cent variation) in 3.

Sulfobromophthalein clearance was not significantly altered by anesthesia. During hypercapnia, however, the mean C_{BSP} fell from 302 to 234 ml per minute, a reduction of 22 per cent ($p < 0.02$). The data in Table III for Subjects 11, 12 and 13 suggest that the impairment of BSP removal by high carbon dioxide levels is not rapidly reversible.

DISCUSSION

No statistically significant alteration in splanchnic hemodynamics was observed in these subjects during anesthesia with thiopental and nitrous oxide maintained by controlled respiration after intravenous administration of succinylcholine. This finding appears to be at variance with the results of previous studies in which hepatic blood flow was observed to decrease during anesthesia with cyclopropane (1), thiopental-nitrous oxide (1) or thiopental-cyclopropane with *d*-tubocurarine paralysis (2). Superimposition of hypercapnia upon the anesthetic state resulted, however, in a statistically significant increase in splanchnic vascular resistance in association with a reduction in estimated hepatic blood flow except in those instances in which the blood pressure rose. Since hypercapnia is difficult to detect clinically without direct measurement, and since it may have occurred in the previous work owing to the use of larger doses of thiopental or to the combination of curare and cyclopropane, the earlier findings may be attributable—at least in part—to undetected hypercapnia. Hypoxia may have also been present, especially when nitrous oxide was used. This uncertainty points up the need for precise monitoring and control of respiratory homeostasis during physiological investigation in anesthetized animals or man.

In the present study repeated determinations of carbon dioxide and oxygen concentrations in the blood provided a reliable basis for the appraisal of the response to anesthesia and hypercapnia separately, without need to allow for the possibility of hypoxia. Nevertheless, the findings must be interpreted with caution. Narcosis or light anesthesia may interfere with circulatory reactivity without changing the pattern of adjustment at rest. Thus, a striking susceptibility to orthostatic hypotension develops following the intravenous administration of morphine or thiopental in doses too small to affect the arterial pressure in the recumbent position (16, 17). The interference with autonomic regulation implicit in this phenomenon might be expected, on the one hand, to minimize reactions that depend primarily upon normal autonomic mediation, and on the other hand, to predispose to excessive nonspecific responses that may dominate the results. Every precaution was taken throughout the course of this study to avoid shifts in position, vascular compression or environmental factors that might have had a disturbing influence. The character

of the changes in the systemic circulation observed during hypercapnia strongly suggests that the anesthetic state did not, in fact, interfere, and that the data may be assessed in terms of the action of carbon dioxide alone.

Coincident with the rise in average arterial P_{CO_2} from a control level of 38 to 56 mm Hg, the arterial pressure tended to rise, the pulse rate to quicken, and the skin of the face and torso to flush. Anesthesia may have diminished the response, and it is also possible that absence of hyperventilation had a moderating effect. In any case, both vasoconstriction and vasodilation were obviously demonstrable, presumably indicating continued operation of the alleged cerebral and local reactions to carbon dioxide. Within the splanchnic vasculature vasoconstriction predominated. Calculated splanchnic vascular resistance increased in almost every subject even when arterial pressure rose sufficiently to maintain or increase splanchnic blood flow. Under these circumstances, the change in resistance may be construed with some confidence as evidence of active arteriolar constriction. The reduction in circulating splanchnic blood volume could also be attributed to this response.

The pressure responsible for the filling of the splanchnic venous reservoir depends in the first approximation upon the splenic, mesenteric, and hepatic arteriolar resistances. Constriction of mesenteric and splenic arterioles would lower the portal venous pressure with a resultant automatic shift of blood into the systemic circuit. Hypercapnia has been reported, however, to raise portal venous pressure and to increase the intestinal venous tone in the experimental animal (18). It may be inferred, therefore, that a similar change occurs in man and that splanchnic venoconstriction is largely responsible for the decrement in volume.

The vasoconstrictive response to hypercapnia appears to be mediated through the central nervous system. Several investigators (19–21) have shown that an increased tension of carbon dioxide in the blood perfusing splanchnic blood vessels elicits a constrictive response only if the splanchnic nerve supply is intact. Following denervation, an elevated Pa_{CO_2} regularly induces dilatation. Evidently this reflex pathway, unlike those concerned in adjustment to posture and pain, is relatively unaffected by neuromuscular blockade and general anesthesia—at least at the levels that were employed in this study. Whether high concentrations of carbon dioxide could overcome the opposing reflex impulses and dilate the vessels by direct action remains unsettled, although Brickner, Dowds, Willitts and Selkurt (22) have shown that mesenteric vasodilation may occur in the intact animal breathing gas mixtures that contain more than 8 per cent carbon dioxide.

The fall in sulfobromophthalein clearance (often in association with diminished extraction) may have been due to direct action of carbon dioxide upon the hepatic parenchymal cells. More work is required to define this phenomenon in terms of the mechanisms of BSP transfer (23) and to evaluate the role of additional side effects of carbon dioxide. Among these, the release of catecholamines may be particularly important, since epinephrine and norepinephrine may be active both in complicating the hemodynamic changes and in affecting hepatocellular activity. Although thiopental anesthesia alone does not increase the plasma concentration of catecholamines, other agents, such as cyclopropane, do (24). It is possible, therefore, that hypercapnia may elicit a more profound or a totally different reaction during anesthesia by drugs other than thiopental-nitrous oxide. Just as hypercapnia may make it difficult to unravel the changes produced by anesthesia, so anesthesia may complicate the understanding of hypercapnia.

SUMMARY

Estimated hepatic blood flow (sulfobromophthalein method), splanchnic blood volume (regional dilution method), calculated splanchnic vascular resistance, and mean arterial pressure did not change significantly or consistently during light general anesthesia with thiopental and nitrous oxide, maintained by mechanically controlled artificial respiration following neuromuscular blockade with succinylcholine. Hypercapnia (Pa_{CO_2}, 56 mm Hg on the average) under these circumstances resulted in a statistically significant increment in mean calculated splanchnic vascular resistance. Estimated hepatic blood flow decreased, remained unchanged or rose depending upon the behavior of the arterial pressure. The average value for the circulating splanchnic blood

volume also decreased significantly. These changes were ascribed to a combination of arteriolar and venous constriction. Sulfobromophthalein clearance and extraction by the liver, although unaffected by anesthesia, decreased significantly during hypercapnia.

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SUSCEPTIBILITY OF RATS TO EXPERIMENTAL PYELONEPHRITIS DURING POTASSIUM DEPLETION *

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It has been adequately shown that potassium depletion can produce functional and structural changes of the kidney both in the experimental animal and in man (1, 2). These changes are primarily tubular and may be partially or wholly reversible. Clinical data suggest an association between potassium nephropathy and pyelonephritis (3–5), and there is evidence both for (5, 6) and against such an association in the experimental animal (7). If such an association were confirmed in the human, it would be one of the most serious of all of the consequences of potassium depletion. A recent study carried out in this laboratory clearly demonstrated increased susceptibility of rats to experimental pyelonephritis *following recovery* from potassium depletion (6). The present investigation was designed to study the susceptibility of rats to pyelonephritis *during* acute potassium depletion.

MATERIALS AND METHODS

White, male, Sprague-Dawley strain rats, weighing 184 to 264 g were used. Potassium depletion was produced by means of a basal diet deficient in potassium, sodium, phosphorus and chloride. Control groups received the following daily quantity of supplementary electrolytes by gavage: 5 ml of gavage solution containing (in millimoles) 2 of potassium, 2 of sodium, 2.5 of chloride and 0.75 of phosphorus. The potassium-deficient groups received (in millimoles) 4 of sodium, 2 of bicarbonate, 0.5 of chloride and 0.75 of phosphorus. The method of producing potassium depletion, including content of diet, and the chemical methods used, have been previously described (6, 8). The animals were "pair-fed" by groups; i.e., the intake of the control groups was limited each day to the average intake of the experimental groups.

Experimental design. The experimental design is shown in Table I. After having been on a potassium-deficient regimen for 2 weeks, the animals of Group A were sacrificed for control analyses, while the animals of Groups B and C were injected with approximately 100 million *Escherichia coli* and continued on a modification of the original regimen which consisted of the same electrolyte-free diet and daily gavage with 5 ml of a solution containing, in millimoles (for controls) 1 of sodium, 2 of potassium, 2.25 of chloride and 0.375 of phosphorus; and (for experimentals) 1 of sodium, 0.125 of chloride, 0.5 of bicarbonate, and 0.186 of phosphorus. This was done in order to maintain the potassium-deficient state without its becoming so severe as to cause death of animals.

Bacteriologic techniques. The strain of *E. coli* used for intravenous inoculation and the details of bacteriological techniques used were exactly the same as those previously described in detail (6). In addition to the studies of renal tissue, other organs were consistently inspected for evidence of infection, and samples of liver and lung from each animal were cultured. Animals of Group B were sacrificed 1 week after injection of *E. coli*, while those of Group C were sacrificed 3 weeks after injection. Sacrifice was so spaced because of the work load involved in dealing with the large number of animals; in addition, the time interval would provide the opportunity to determine whether bacterial multiplication in the kidney was increasing or decreasing during the postinjection period. Five experimentals and an equal number of controls from both Groups B and C had analyses of sera and skeletal muscle in order to document and determine the degree of potassium depletion.

TABLE I

Experimental design

Group	Description	No. of exptl. rats	No. of control rats
A	K-depletion 2 wks; analysis of renal histology, serum electrolytes, muscle K; culture for spontaneous infection	5	5
B	K-depletion 2 wks; sacrificed 1 wk after injecting 100,000,000 *E. coli* i.v.; analysis of serum electrolytes and muscle K in a representative sample	22	21
C	K-depletion 2 wks; sacrificed 3 wks after injecting 100,000,000 *E. coli* i.v.; analysis of serum electrolytes and muscle K in a representative sample	17	17

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† Markle Scholar in Medical Science.

RESULTS

Condition of animals. Six rats died as a result of gavage during the first week of the study and four were found dead in their cages during the first 48 hours after injection of *E. coli.* These were excluded from the study. The remainder appeared reasonably healthy and there was no difference between the final average weight of experimental and control groups (241 g vs 243 g). The results of the chemical determinations are shown in Table II. Analyses of serum electrolytes of the three experimental groups as compared with their controls revealed a hypokalemic, hypochloremic alkalosis. Serum sodium concentrations remained normal. There was a moderate decrease in skeletal muscle potassium content when it was measured in terms of milliequivalents of potassium per 100 g of fat-free dry solids. The degree of potassium depletion increased slightly during the three week postinjection period as is seen in a comparison of the data from the three groups. As discussed by Oliver and co-workers (2), there appears to be an element of tubular obstruction in potassium nephropathy, and tubular dilatation consistent with this was microscopically evident at three and five weeks (Groups B and C). Tubular dilatation was not yet clearly evident at two weeks (Group A) when the animals were injected with *E. coli.* The results pertaining to renal infection are shown in Table III. Gross appearance, colony counts for the pooled halves of each of the kidneys, and microscopic appearance of the two remaining halves are presented. Ten of the 39 potassium-depleted animals, but only 1 of the 38 controls had the combination of gross abscess, colony count over 100,000 *E. coli* per g of kidney, and unequivocal microscopic evidence of pyelonephritis ($p < 0.02$).[1] Additionally, in the potassium-depleted groups: 2 animals had unequivocal microscopic evidence of pyelonephritis without gross or cultural evidence; 2 had macroscopic abscesses and high colony counts with only small round cell infiltrates microscopically; 2 had macroscopic scars combined with microscopic evidence of pyelonephritis (one of the latter had a high colony count of *E. coli* in the kidneys, while the other had a count of 1,400 *E. coli* per g of kidney); and 1 had macroscopic and microscopic evidence of infection with a colony count of 4,000 *E. coli* per g of kidney. In the control groups, 5 additional rats had positive evidence of infection in one or more of the three descriptive categories but not in all three. If all of these additional animals are included, 17 of 39 potassium-depleted and 6 of 38 controls had pyelonephritis ($p < 0.02$). Eleven of the ex-

[1] A chi-square test was used to calculate p values.

TABLE II

Analyses of sera and skeletal muscle

Group	K-depleted			Controls		
	Serum		Muscle	Serum		Muscle
	K	CO_2	K	K	CO_2	K
	mEq/L	*mEq/L*	*mEq/100 g FFDS **	*mEq/L*	*mEq/L*	*mEq/100 g FFDS **
A	2.2	27.5	37.4	3.8	21.0	43.9
	3.1	19.8	41.1	4.5	24.5	43.9
	2.8	26.1	37.7	4.1	17.7	47.6
	3.5	28.4	37.5	4.3	23.1	48.0
	3.1	27.0	37.5	4.1	21.7	47.2
B	3.0	27.0	35.8	4.9	23.8	45.8
	2.8	34.2	32.5	5.4	24.7	47.7
	4.1	31.2	42.6	5.1	25.5	49.0
	4.0	35.2	36.7	5.0	30.1	46.7
	3.3	32.7	39.7	5.5	25.8	46.6
C	2.4	32.8	30.5	4.1	24.8	44.3
	2.4	28.9	34.1	4.7	26.7	43.6
	2.6	33.5	33.0	4.9	25.8	45.2
	3.2	29.3	39.7	4.5	21.2	44.7
	2.6	30.4	33.0	4.1	21.4	45.7

* FFDS = fat-free dry solids.

TABLE III

Colony counts and description of kidneys after injection of E. coli

	K-depleted			Controls		
Group	Gross appearance *	Colony count	Microscopic †	Gross appearance	Colony count	Microscopic
		E. coli/g kidney			*E. coli/g kidney*	
B	*	4,600,000	†		0	
		0			0	
		0	†		1,000	
		0			0	
		10,000			0	
		0			0	
		4,000			0	
		0			0	
	*	100,000,000	†		0	
		0			0	
	*	300,000	†		0	
	*	2,000,000	†		0	
	*	280,000		*	150,000	†
		0	†		0	
	*	100,000	†		0	
		0			0	†
	*	16,000,000	†	*	9,100	†
		0			0	
		0			0	
	*‡	140,000,000	†	*	3,300,000	
	*	10,000,000	†		0	
		0				
C	*	110,000	†		0	
	scars	100,000,000	†	*	0	†
		0			0	
		0			0	
		0			0	
	*	4,000	†		0	
	*	2,300,000			0	
		0			0	
		0			0	
	scars‡	1,400	†	*	0	†
		0			0	
	*	100,000,000	†		0	
	scars‡	0			0	
		0			0	
		0			0	
	*	30,000			0	
		0			0	

* Macroscopic abscess.
† Unequivocal microscopic evidence of infection.
‡ Lung abscess also, due to *E. coli*.

perimental group and 9 of the controls had *minute* collections of round cells, usually adjacent to blood vessels, unaccompanied by macroscopic or cultural evidence of infection, and were considered uninfected.

Occasional discrepancies between cultural and histological evidence are to be expected, since the lesions are focal, and since each kidney is divided into halves, one of which is homogenized for culture and one of which is fixed for sectioning. Also, of course, histological evidence of infection may persist indefinitely after the lesion becomes sterile.

Three of the experimentals, but none of the controls, had positive cultures of liver or lung. One of the former had an abscess of the lung.

It is apparent that the *E. coli* infection tends to "burn out" with the passage of time, since in Group B, sacrificed at one week, 9 of 22 experimentals and 2 of 21 controls had colony counts of 100,000 per g of kidney or greater, while in Group C, sacrificed at three weeks, only 4 of 17 experimentals and no controls had such colony counts. However, there was no significant difference in the incidence of infection between Group B and C experimentals, when the positive state of

any one or more of the diagnostic criteria is taken to mean infection.

DISCUSSION

In a recent review of the literature (9), a total of 14 patients with potassium depletion showed evidence suggesting pyelonephritis, as judged by history, urine cultures or renal histology. In at least three of these, the pyelonephritis may have preceded the potassium depletion. The cause of the potassium depletion was primary aldosteronism in eight of the patients and was due to gastrointestinal causes in five.

In the experimental animal, there is evidence both for and against the association of pyelonephritis and potassium nephropathy. One possible explanation for this discrepancy is the fact that the use of more virulent bacteria may permit the demonstration of slight increases in susceptibility, whereas the use of less virulent bacteria would not. Since the strain of *E. coli* used in this laboratory will infect some normal rats if it is carried through a number of "passages" in rats with ligated ureters and if it is injected in large doses, it is reasonable to conclude that a relatively virulent organism is being used.

SUMMARY

Rats with *acute* potassium nephropathy have been shown to exhibit increased susceptibility to experimental pyelonephritis. The factor responsible for this increased susceptibility is unknown, but the authors believe that the most likely one is tubular obstruction or "internal hydronephrosis."

ACKNOWLEDGMENT

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EFFECTS OF PLASMAPHERESIS ON ALBUMIN POOLS IN RABBITS

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The normal regulation of intravascular plasma protein mass has not been fully investigated. It has long been known that plasma proteins rapidly return to normal concentrations after plasmapheresis, but the means by which this takes place has not been clearly demonstrated. In pathological states, such as nephrosis and hypercatabolic hypoproteinemia, where there may be a steady loss of plasma protein, some compensatory mechanism (see next paragraph) must be involved in cases in which the intravascular plasma proteins are in a steady state. Frequently the protein concentration alone is determined, so that it is not known whether changes are due to variations in plasma volume or in total intravascular protein mass. A steady state can only be said to exist when the plasma protein synthesized is equal to that catabolized, so that the total mass of protein, but not necessarily the concentration, is constant.

Disturbances of intravascular protein mass could be compensated for by *a*) change in synthesis rate, *b*) change in catabolic rate, *c*) net transfer of protein between extravascular and intravascular protein pools or *d*) a combination of several of these. There is also the possibility that the proportion of the different protein fractions is altered and that loss of one fraction is replaced by an increase of another fraction.

The purpose of this investigation was to obtain further evidence on this problem by removing protein by plasmapheresis and observing the effect on the catabolic rate, intravascular pool mass, concentration and volume, and on the sum of synthesis rate plus net transfer rate from extravascular to intravascular pool. Rabbits on a normal diet were used, and a single protein fraction, albumin, was considered.

I^{131}-rabbit albumin was injected intravenously into rabbits, and plasma albumin specific activity (microcuries per gram), total body radioactivity and urinary radioactivity were measured. It is assumed throughout that I^{131}-albumin behaves as normal rabbit albumin (1, 2), that after catabolism the I^{131} is rapidly excreted, and that there is rapid mixing in each separate protein pool so that within any one pool the specific activity is uniform.

A control period of ten days from the injection was allowed before starting plasmapheresis. The normal catabolic rate and pool masses were thus determined for each animal. Daily determinations of intravascular protein mass, concentration and volume were also made throughout each experiment in a control animal not subject to plasmapheresis.

Intravascular protein mass and plasma volume were determined by isotope dilution. Since the proportion of albumin to total plasma protein was found to be constant throughout the experiment, albumin masses could be calculated from total protein masses.

METHODS

Theoretical

Extravascular pool. During the control period, the extravascular protein mass was determined by the equilibrium time method (3–5). In this method the ratio of extravascular to intravascular radioactivity, when extravascular activity is maximal, is taken to be the ratio of extravascular to intravascular protein. The assumptions made, in addition to those listed above, are that the pool masses and rates of exchange and catabolism are constant and that newly synthesized protein is discharged into, and catabolism occurs in, the intravascular pool or in a pool which is in rapid exchange with it. Extravascular activity is found by subtracting total intravascular activity (i.e., specific activity × mass) from total body activity.

During the plasmapheresis period the pools are probably not in a steady state so that this method is not valid.

Catabolic rate. The albumin catabolic rate is measured in grams per day by the method described by Campbell, Cuthbertson, Matthews and McFarlane (3). The 24-hour urinary activity in microcuries is divided by the mean plasma albumin specific activity in microcuries per gram during the 24 hour period. This assumes that catabolism takes place in the intravascular pool or in some pool in rapid exchange with it (6). This method is valid even when the catabolic rate and pool mass are varying, since

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the only assumption involved is that the specific activity of the albumin catabolized is the same as that of the plasma albumin so that in any given period:

$$\frac{\text{activity in urine}}{\text{mass of albumin catabolized}} = \frac{\text{activity in plasma}}{\text{mass of albumin in plasma}}$$

and therefore

$$\frac{\text{activity in urine}}{\text{plasma albumin specific activity}} = \text{mass of albumin catabolized.}$$

Synthesis rate. Direct measurement of synthesis rate from incorporation of labeled amino acids into plasma proteins requires a knowledge of the immediate precursor specific activity, and this is very difficult to obtain (7). However, variations in synthesis rate give rise to variations in specific activity of labeled proteins. Catabolism alone will not alter the specific activity of the remaining labeled protein, but the extent to which the label is diluted will depend upon the rate of entry of new unlabeled molecules into the system of pools. Tarver (8) tabulates the effects of various changes in the system. However, in general, it cannot be assumed that the specific activities in the different pools are the same (3, 4), and exchange between the pools is therefore a complicating factor.

Consider a system of an intravascular and one extravascular protein pool with catabolism in the intravascular pool and newly synthesized protein entering this pool.

Following the notation of Rescigno (9) and Matthews (4), let subscript 1 denote intravascular pool, and subscript 2 the extravascular pool.

X_1, X_2 = fraction of injected dose in pools 1, 2 at time t.
M_1, M_2 = mass of protein in pools 1, 2.
K_{01} = protein synthesis rate as a fraction of M_1 per day.
K_{12} = protein catabolic rate as a fraction of M_1 per day.
K_T = net rate of transfer of protein from pool 2 to pool 1 as a fraction of M_1 per day.
K_{12} = fraction of protein in pool 1 entering pool 2 per day.
K_{21} = fraction of protein in pool 2 entering pool 1 per day.

Then: slope of intravascular specific activity curve (i.e., 0.693/half-life in days)

$$= \frac{-\,d(X_1/M_1)/dt}{X_1/M_1} = K_{01} + K_{21}\frac{M_2}{M_1} - K_{21}\frac{X_2}{X_1} = b.$$

Slope of extravascular total activity curve

$$= \frac{-\,dX_2/dt}{X_2} = K_{21} - K_{12}\frac{X_1}{X_2} = c$$

$$K_{01} = b + \frac{X_2}{X_1}c + K_{12} - K_{21}\frac{M_2}{M_1}$$

$$K_T = \frac{-\,dM_2/dt}{M_1} = K_{21}\frac{M_2}{M_1} - K_{12}$$

$$\therefore\ K_{01} + K_T = b + \frac{X_2}{X_1}c. \qquad [1]$$

This equation is always valid even if all rates and masses are varying. For a steady state, M_2 is constant and $K_T = 0$, so that $K_{01} = b + (X_2/X_1)\,c$. Equation 1 was used to find the synthesis rate + transfer rate. Unfortunately it is not possible to distinguish between changes in synthesis rate and changes in transfer rate. It can be seen from Equation 1 that if b, c, and X_2/X_1 are constant, then $K_{01} + K_T$ must be constant.

Also: slope of total body activity curve

$$= \frac{-\,d(X_1 + X_2)/dt}{X_1 + X_2} = K_{12}\frac{X_1}{X_1 + X_2} = d.$$

$\therefore$ If $b = c = d$ (i.e., intravascular specific activity, extravascular total activity and total body activity curves are all parallel) then:

$$K_{01} + K_{21}\frac{M_2}{M_1} - K_{21}\frac{X_2}{X_1} = K_{21} - K_{12}\frac{X_1}{X_2}$$

$$= K_{12}\frac{X_1}{X_1 + X_2}. \qquad [2]$$

Fractional rate of change of intravascular pool mass

$$= \frac{dM_1/dt}{M_1} = K_{01} - K_{12} - K_{12} + K_{21}\frac{M_2}{M_1} \qquad [3]$$

Combining Equations 2 and 3, it can easily be shown that;

$$\frac{dM_1/dt}{M_1} = 0$$

Therefore, if intravascular specific activity, extravascular total activity and total body activity curves are parallel, the mean intravascular protein mass must be constant.

Experimental

Animals. Male Sandylop rabbits bred at the National Institute for Medical Research were used; they received pelleted diet SG1 (10). The drinking water contained 0.005 per cent sodium iodide and 0.47 per cent sodium chloride from 3 days before the first injection until the end of the experiment, in order to increase the rate of iodide excretion.

Preparation of rabbit albumin. Rabbit albumin was kindly prepared by Dr. S. Cohen from 4 ml rabbit serum by electrophoresis, according to Porath (11), on a column of treated cellulose 3 cm in diameter and 45 cm long, using a current of 22 ma for 46 hours, and a borate phosphate buffer (pH 8.60, $\mu = 0.05$).

Iodination. Iodination was carried out by the iodine monochloride method as described by McFarlane (12), after which free iodine was removed by passage through anion exchange columns of Deacidite (Permutit & Co., London).

Injection and samples. The iodinated albumin or plasma, in a volume of less than 2 ml, was injected into the marginal ear vein, and a sample of blood was withdrawn about 5 minutes later from the opposite marginal ear vein. The activity to be injected and the syringe washings were counted and the injected dose obtained by difference.

Urine was collected every 24 hours, before I^{132} injection, the cages being washed down with 0.05 N sodium hydroxide. The volume of urine and washings was measured and a 3 ml aliquot taken for counting.

TABLE I

Fall in intravascular specific activity after injection of labeled protein, before and after plasmapheresis

Time from injection	Relative specific activity		
	Before plasmaph.	2 hrs after plasmaph.	4.3 hrs after plasmaph.
5 min	100	100	100
1.2 hr	90.1		
2.0 hr		88.0	
2.2 hr	78.9		
2.8 hr			82.5

Intravascular protein mass and plasma volume. Intravascular protein mass and volume were obtained by isotope dilution, initially from the injection of I^{131}-albumin, and subsequently throughout the experiment by injection of I^{132}-rabbit plasma. When I^{132}-plasma was injected, a blood sample was taken a few minutes before the injection to determine the I^{131} activity in the blood at this time. Another sample was then taken about 5 minutes after injection; the injected dose divided by the I^{132} activity per milliliter of plasma and per gram of protein gave the plasma volume and intravascular total protein, respectively. Since the fall in specific activity after injection is slow, it was considered sufficient to take a single sample for specific activity measurement (see Table I).

TABLE II

Nonprotein-bound radioactivity before and during plasmapheresis

	Time	Activity in TCA supernatant
	hrs	*% of total*
Plasma	0.083 (5 min)	0.69
	17.7	1.25
	26	1.07
	40	0.87
	50	0.79
	64	1.11
		*
	68	1.97
	89	0.92
		*
	91	1.29
	113	0.79
		*
	116	1.40
	137	1.03
	185	1.06
		*
	187	1.44
	209	0.92
		*
	212	1.28
		*
	233	0.81
		*
	235	0.94
	257	1.04
		*
Lymph	261	>1.1 and <2.1

* Plasmapheresis.

Measurement of radioactivity. Urine samples and dilutions of plasma samples were counted in a well-type scintillation counter. Since urine samples were collected in the morning before I^{132} injection, the I^{131} injected the day before had decayed to a negligible amount compared with the I^{131} activity.

Plasma samples containing both I^{132} and I^{131} activity were counted as soon as possible and then recounted the following day to obtain the I^{131} activity. By subtracting the I^{131} activity (corrected for decay) from the first count, the I^{132} activity at this time was obtained.

The total body activity was measured in a ring counter of eight Geiger tubes (4). The I^{131} and I^{132} activities for injection and the activity in the washings were also measured in the ring counter.

Iodine-132. Te^{132} on an alumina column was obtained from A.E.R.E.,[1] Harwell. I^{132} was eluted daily by pouring 0.01 M ammonia through the column. The eluent was evaporated down to a small volume which was used for iodination. A sample of each eluent was counted in the ring counter and recounted the next day to check the proportion of I^{131} activity. This was always less than 0.5 per cent.

Protein determinations. Total protein was determined by the biuret method of Gornall, Bardawill and David (13). The proportion of albumin to total protein was determined by finding the ratio of I^{131}-albumin to I^{131}-total protein

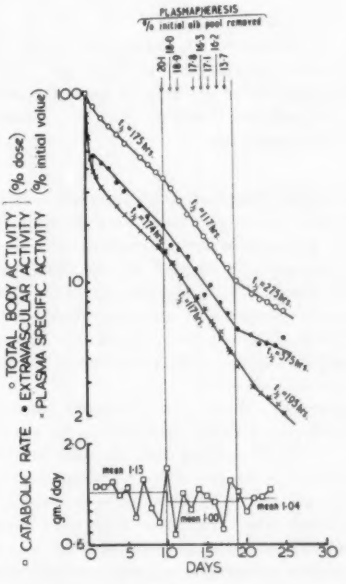

FIG. 1. EXPERIMENT C0, RABBIT A. Total body activity, ○; total extravascular activity, ●; plasma albumin specific activity as per cent of value 5 minutes after injection, ×; albumin catabolic rate, □ before, during and after plasmapheresis.

[1] Atomic Energy Research Establishment.

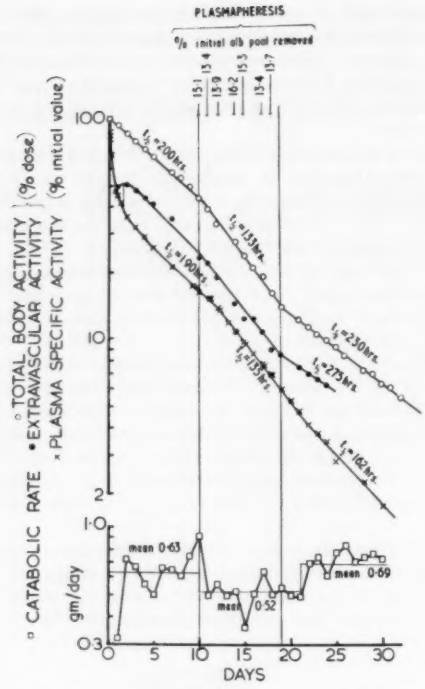

FIG. 2. EXPERIMENT C1, RABBIT B. Total body activity, ○; total extravascular activity, ●; plasma albumin specific activity as per cent of value 5 minutes after injection, ×; albumin catabolic rate, □ before, during and after plasmapheresis.

specific activities, since only the albumin was labeled with I^{131}. Albumin specific activities were determined as follows. The globulins were precipitated with 1 per cent trichloroacetic acid in alcohol as described by Debro, Tarver and Korner (14). The albumin in the supernatant was then reprecipitated with an equal volume of ether and the precipitate dissolved in 2 ml saline. This was then counted and the albumin determined by the biuret method, as for the plasma samples.

Plasmapheresis. Blood was withdrawn from the marginal ear vein into a sterile tube containing about 500 IU of heparin. After centrifugation, the supernatant plasma was removed, and the red cells and trapped plasma were diluted with about 5 to 10 ml of saline and reinjected into the marginal ear vein. The interval between bleeding and injection of red cells was approximately 40 minutes. In each experiment plasmapheresis was continued daily for 9 days except on Day 4 in Experiment C0 and on Days 4 and 7 in Experiment C1.

Nonprotein-bound activity. This was determined by adding 1 drop 10 per cent sodium iodide, 0.5 ml 10 per cent albumin solution and one-third of the volume of 30 per cent trichloroacetic acid. The percentage of radioactivity in the supernatant was taken as the percentage of nonprotein-bound activity. The results for one rabbit are shown in Table II.

RESULTS

The complete experiments described were carried out with plasmapheresis in four rabbits and in two control rabbits. There were also incomplete experiments in three other rabbits in which only one isotope was used. The results were all similar.

Figures 1 and 2 show the total body activity, extravascular activity, and plasma specific activity curves and also the catabolic rates for two of the rabbits. Plasma specific activity curves are seen to be approximately parallel to total body activity curves before and during the period in which daily plasmapheresis was carried out, which implies that the mean intravascular protein mass is constant during this period (see Equation 3 et seq.). After this "plasmapheresis period" the slope of the plasma specific curve is not constant but is greater than that of the total body activity curve. The changes in plasma specific activity slope sometimes lag behind the changes in total body activity slope.

The catabolic rate falls slightly during the plasmapheresis period and rises again afterward. Figure 3 shows the changes in intravascular specific activity after plasmapheresis in greater detail.

Figure 4 shows the variations in intravascular protein mass, concentration and volume for one of these rabbits. There is a gradual fall in mean concentration during the plasmapheresis period, with a gradual return to normal afterward. This mainly reflects an increase in plasma volume as the total protein mass is approximately constant. (The mean intravascular protein mass during the

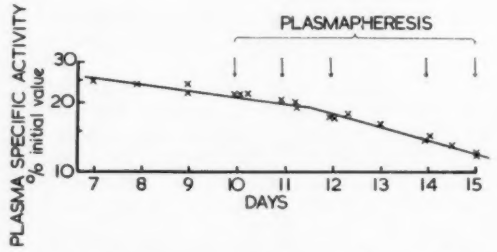

FIG. 3. EXPERIMENT C0, RABBIT B. Plasma albumin specific activity as per cent of value 5 minutes after injection, before and during plasmapheresis.

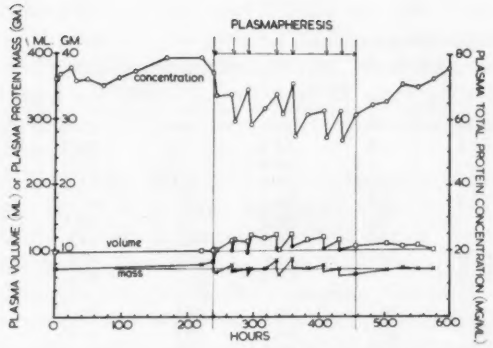

FIG. 4. EXPERIMENT C1, RABBIT B. Intravascular total protein mass, ×; intravascular total protein concentration, ○; plasma volume, □ before, during and after plasmapheresis. Calculated protein masses after plasmapheresis, ●; these are the values before plasmapheresis minus the protein withdrawn.

plasmapheresis period is actually 6.5 per cent less than in the control period in this rabbit. This is probably significant, $p \sim 2$ per cent). The concentration falls sharply immediately after plasmapheresis and later rises. Figure 5 shows the variations in intravascular protein mass, concentration and volume for a control rabbit with no plasmapheresis.

Table III shows the pool masses and the changes in catabolic rate, and synthesis and transfer rate calculated as described above. The total compensatory changes agree quite well with the amount of albumin withdrawn. This must be so if the

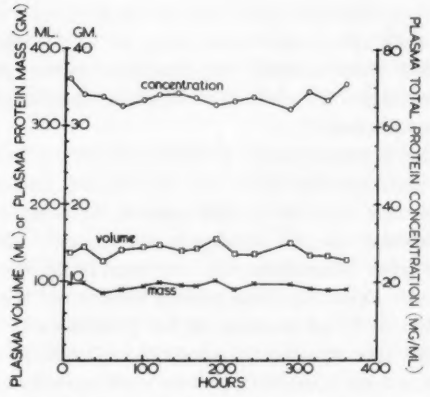

FIG. 5. EXPERIMENT C1, RABBIT D, CONTROL. Intravascular total protein mass, ×; intravascular total protein concentration, ○; plasma volume, □.

TABLE III

Effect of plasmapheresis in rabbits

Expt.	Rabbit	Weight Before expt.	Weight After expt.	Initial intravasc. albumin	Initial extravasc. albumin	Total alb. withdrawn	% Initial Intravascular Albumin Per Day: Catabolic rate Plasmapheresis period Before	Catabolic rate Plasmapheresis period During	Catabolic rate Plasmapheresis period After	Synthesis+transfer rate Plasmapheresis period Before	Synthesis+transfer rate Plasmapheresis period During	Synthesis+transfer rate Plasmapheresis period After	Decrease, catabolic rate	Increase, synthesis+transfer rate	Total compensatory changes	Total alb. withdrawn
		kg		*g*	*g*	*g*							—During plasmapheresis period—			
C0	A	2.72	2.96	5.04	7.30	6.85	22.4	19.9	20.7	23.5	35.4	20.0	2.5	11.9	14.4	15.1
C0	B	2.08	2.52	4.65	6.26	4.04	18.4	16.8	15.2	17.0	25.6	18.6	1.6	6.6	8.2	9.2
C1	A	2.44	2.62	4.41	5.35	5.85	20.6	15.0	19.0	22.4	29.7	20.4	5.6	6.3	11.9	14.7
C1	B	2.33	2.54	3.72	4.76	4.36	17.0	14.0	18.5	22.4	29.9	21.2	3.0	6.6	9.7	11.2

TABLE IV

Intravascular albumin as per cent total intravascular protein *

Experiment	C0, A		C0, B		C1, A		C1, B	
	hrs	*% alb.*	*hrs*	*% alb.*	*hrs*	*% alb.*	*hrs*	*% alb.*
Before plasmapheresis	0	64.6	0	54.4	0	53.4	0	50.9
					1.3	56.9	1.3	51.1
					22.5	57.8	22.5	61.0
	48	64.4	48	58.5	47	54.4	47	47.0
					95	60.7	95	55.5
					167	58.5	167	51.6
	240	66.9	240	61.0	239	58.0	239	49.6
	Mean	65.3		58.0		57.1		52.4
During plasmapheresis					267	57.1	267	52.4
	288	61.1	288	58.7	291	58.5	291	48.0
	364	65.0	364	52.4	339	60.5	339	50.5
					435	62.0	435	55.9
	457	52.1	457	59.5	456	48.4	456	52.4
	Mean	59.4		56.9		57.3		51.8
After plasmapheresis	552	62.5	576	54.1	528	52.5	528	53.0

* Ratio of total protein specific activity to albumin specific activity.

assumptions made are correct, and confirms that the intravascular albumin mass is approximately constant. About 70 to 80 per cent of the withdrawal is compensated for by an increase in the synthesis plus transfer rate and the remainder by a decrease in the catabolic rate. The greatest decrease in the catabolic rate is 27 per cent of the initial value. Table IV shows the ratio of total protein to albumin specific activity. Since this ratio does not change significantly throughout the experiment the albumin pool must be a constant fraction of the total protein pool.

Since the total intravascular albumin mass is constant, changes in percentage of catabolic and synthesis rates are proportional to changes in absolute rates (i.e., in milligrams per day).

DISCUSSION

Removal of intravascular protein by plasmapheresis appears to be mainly compensated for either by a change in the synthesis rate or by transfer of protein from extravascular to intravascular pool, or both. These possibilities cannot be distinguished since only the synthesis plus transfer rate can be determined. The change in catabolic rate is comparatively small. The plasma volume increases after each bleed and the concentration falls. Later, concentration rises, so that the protein is evidently replaced more slowly than is the water. An increase in plasma volume after plasmapheresis was also observed by Whipple, Miller and Robscheit-Robbins (15), and by Rodionov and co-workers (16).

If the synthesis rate remains constant, the extravascular pool must be greatly reduced. For example, for Rabbit A in Experiment C0:

> (albumin withdrawal rate − decrease in catabolic rate) × 9 days = 78 per cent extravascular pool.

I.e., this pool would have decreased to 22 per cent of the initial value. This seems very unlikely. The concentration of total protein for this rabbit fell to about 78 per cent of the original value, so that unless the extravascular volume was reduced, the concentration gradient between intra- and extravascular pools would have to be 3.5 times greater than normal. It therefore seems very probable that the synthesis rate is increased during plasmapheresis.

The constant ratio of extravascular to intravascular activity does not necessarily indicate a constant ratio of protein masses, since the extravascular specific activity is not known. Similarly after plasmapheresis, the increasing extravascular to intravascular activity ratio is not necessarily due to an increase in the protein ratio, although this remains the simplest explanation. If there is a net transfer of protein from intravascular to extravascular pool after the plasmapheresis period, the transfer rate K_T will be negative, and the synthesis rate will be greater than the values cal-

culated for synthesis plus transfer rate (see Table III). Thus, by this explanation the synthesis rate would remain higher than normal after the plasmapheresis period is over and the extra protein produced would increase the extravascular pool mass.

If extravascular mass remains constant throughout, i.e., if the transfer rate is zero, then the synthesis rate must have returned to normal after the plasmapheresis period (see Table III). The system would then be in equilibrium, except that the catabolic rate has not quite returned to normal in most cases. However, the small difference between synthesis and catabolic rate seems hardly great enough to account for the difference in extravascular activity and intravascular specific activity slopes so that there was probably some change in extravascular protein mass. In the steady state the slopes should, of course, be equal (8).

The most likely hypothesis, therefore, seems to be that during the plasmapheresis period the intravascular pool mass is kept constant mainly by both net transfer of protein from the extravascular pool and increased synthesis, and that after the plasmapheresis period the synthesis rate remains high for a time so that the extravascular pool gradually returns to normal. This would be in agreement with the results of some other workers. Wasserman, Joseph and Mayerson (17) conclude from plasmapheresis and infusion experiments in dogs that albumin is first replaced from the extravascular pool and later by increased synthesis. Rodionov and associates (16) reach the same conclusions from plasmapheresis experiments in dogs with and without liver denervation, but they also find a large increase in globulins after bleeding. Reeve and Roberts (18) find a constant catabolic rate of albumin in grams per day per unit body weight for normal rabbits, independent of plasma albumin concentration. They conclude from this that it is likely that albumin mass is maintained by regulation of the synthesis rate.

Experiments in which plasma proteins are depleted by means of a low protein diet (19–21) may not be directly comparable with plasmapheresis experiments, since the depletion is produced in a different way.

It is remarkable how little the mean intravascular pool mass deviates from the normal value considering the large amount of protein withdrawn. A sensitive mechanism must exist for altering the transfer rate plus synthesis rate to compensate for the protein removed. Furthermore, the sum of the transfer rate plus synthesis rate must be constant during the plasmapheresis period (see Equation 3 et seq.). It seems likely that the transfer rate will be reduced as the extravascular pool becomes smaller, so that the synthesis rate must be increasing by just the required amount to balance this effect. This suggests that synthesis rate depends upon the extravascular protein mass or concentration. Thus the intravascular pool would be maintained at the expense of the extravascular pool, and the reduction of this pool would stimulate increased synthesis.

SUMMARY

The effects on the albumin pools of a period of daily plasmapheresis in rabbits are as follows.

1. Intravascular albumin mass fluctuates about a mean value which is approximately constant throughout the experiment.

2. Plasma volume increases during the plasmapheresis period and albumin concentration falls. After the plasmapheresis period, volume and concentration slowly return to normal.

3. Intravascular albumin is a constant fraction of intravascular total protein throughout the experiment.

4. During the plasmapheresis period, intravascular specific activity and total body activity curves are parallel.

5. Approximately 70 to 80 per cent of the albumin removed is replaced by either net transfer of protein from extravascular to intravascular pool or by increased synthesis, or both. The data do not distinguish between these possibilities.

6. Twenty to thirty per cent of the albumin removed is compensated for by a reduction in the catabolic rate.

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ACTIVATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE OF ENZYME-DEFICIENT SUBJECTS. II. PROPERTIES OF THE ACTIVATOR AND THE ACTIVATION REACTION *

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The primary metabolic defect in subjects susceptible to hemolysis by various drugs and fava beans is considered to be the low glucose-6-phosphate dehydrogenase (G6PD) activity in their red cells (1, 2). Such diminished enzymatic activity could be the end result of qualitative or quantitative changes in the enzyme as well as enzyme inhibition or lack of enzyme activation. However, studies on the properties of erythrocyte G6PD in such subjects and in normal subjects revealed no qualitative difference between them (3, 4).

In a previous communication we demonstrated the presence of an activator of G6PD in normal erythrocyte stroma which increased the G6PD activity in the "sensitive" [1] hemolysate (5). This activator was found to be absent in the stroma of "sensitive" erythrocytes. Our recent finding that G6PD activity is also present in red cell stroma made it necessary to reinvestigate the validity of the G6PD activator.

METHODS

Bank blood collected in an ACD [2] solution and stored for about 2 weeks was used for the preparation of stroma. The red cells were washed three times in 0.85 per cent NaCl solution and the red cell stroma was prepared by gradual hemolysis as follows: to 1 vol of packed erythrocytes 9 vol of cold distilled water was added by drops, using a magnetic stirrer. The stroma was obtained by centrifugation at 28,000 G for 30 minutes. The stroma was suspended in 10 vol of 0.15 per cent NaCl solution and centrifuged for 5 minutes. This procedure was repeated about ten times until the supernatant was almost colorless and contained no G6PD activity. The packed stroma was diluted with an equal volume of 0.85 per cent NaCl solution. One ml of such a stroma suspension contains about 30 mg dry weight. The hemolysates were prepared from fresh blood taken in heparin or ACD solution. The red cells were washed three times in 0.85 per cent NaCl solution. The cells were lysed by the addition of 4 vol of water to 1 vol of packed red cells. The stroma was removed from the hemolysates by centrifugation at 28,000 G and the supernatants used.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase (6PGD) were assayed by a slight modification of the method of Kornberg and Horecker (6) and Horecker and Smyrniotis, respectively (7), as used in this laboratory (8).

The presence of G6PD and 6PGD in stroma suspension was demonstrated by the method of Motulsky and Campbell (9), which is based on dye discoloration. The reaction mixture contained 0.4 ml stroma suspension added to 2 ml water. To that were added 0.4 ml of 0.25 M Tris buffer, pH 7.5; 0.5 ml of 5×10^{-3} M glucose-6-phosphate or 6-phosphogluconate; 0.1 mg TPN; and 0.5 ml of 0.03 per cent brilliant cresyl blue. After mixing, the reaction mixture was covered with 1 ml mineral oil and incubated at 37° C. The rate of the dye reduction is directly related to TPNH formation. The latter is formed when either the glucose-6-phosphate or 6-phosphogluconate is oxidized. This is a semiquantitative screening method used to distinguish normal from G6PD-deficient subjects.

Partial purification of G6PD was performed by precipitating the enzyme from a normal or "sensitive" hemolysate with 20 per cent ethanol. The precipitate was suspended in 0.2 M Tris buffer, pH 8.0. The enzyme went into solution and the residue was discarded. By the addition of ammonium sulfate, up to a concentration of 40 per cent (wt/vol) to the solution, the enzyme was reprecipitated. By this method a 20-fold purification based on the total protein content of the hemolysate was achieved. The yield of enzyme obtained was about 60 per cent.

RESULTS

Properties of the activation reaction. The effect of red cell stroma on G6PD activity of red cell hemolysate is summarized in Table I. It is obvious

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[1] Hemolysates and stroma obtained from erythrocytes which exhibit a very low G6PD activity will be referred to as "sensitive."

[2] Acid citrate dextrose, Formula B. National Institutes of Health, USA.

TABLE I

The effect of red cell stroma on G6PD activity in hemolysates

No. of expts.	Reaction mixture*		G6PD activity†
	Stroma	Hemolysate	
14		Normal	14.3 ± 7.9
55		"Sensitive"	1.6 ± 1.1
14	"Sensitive"	Normal	14.2 ± 7.7
7	"Sensitive"	"Sensitive"	1.7 ± 1.7
9	Normal	Normal	15.1 ± 8.9
55	Normal	"Sensitive"	7.5 ± 6.6

* The reaction mixture contained 1 ml of hemolysate added to 1 ml of stromal suspension when indicated. After 15 minutes' incubation the reaction mixture was spun down at 28,000 G for 10 minutes and the supernatant used for the enzymatic assay.

† In Δ A per minute per gram hemoglobin; mean activity ± standard deviation.

that normal red cell stroma has significantly increased the enzymatic activity of sensitive hemolysates (sometimes even to normal levels). No such effect was observed with sensitive stroma. When sensitive and normal stroma were mixed in different proportions the activating capacity of normal stroma was not affected.

The activation reaction progressed with time (Figure 1). It can be seen that under these experimental conditions the activation reached its maximum at about 10 minutes. This reaction was found to be pH-dependent, being optimal at about pH 7.2 (Figure 2), and temperature-dependent (Figure 3).

The effect of metal ions was tested on the activation reaction and on G6PD activity. Figure 4

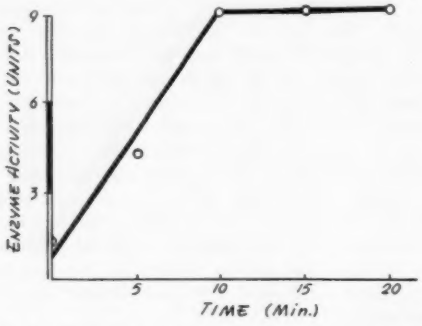

FIG. 1. THE EFFECT OF TIME ON THE ACTIVATION REACTION. The incubation mixture consisted of 1 ml of "sensitive" hemolysate and 1 ml of stroma suspension. Five tubes were incubated at 37°C. At 0 time, 5, 10, 15 and 20 minutes, the reaction mixture was spun down at 28,000 G and the supernatant was tested.

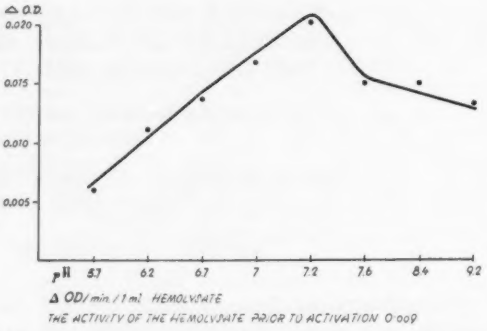

FIG. 2. THE EFFECT OF pH ON THE ACTIVATION REACTION. The incubation mixture consisted of 1 ml of "sensitive" hemolysate, 1 ml of stroma suspension and 2 ml buffer, at the desired pH, with 5 per cent succinic acid solution. The pH of the reaction mixture was tested and the obtained pH of the mixture is indicated in the figure. After incubation, the reaction mixture was centrifuged at 28,000 G for 10 minutes and the supernatants were used for the assay.

represents the effect of mercury on both reactions. As can be seen from the figure, the activation reaction was found to be more sensitive to Hg than the G6PD activity per se. Fifty per cent inhibition of these two activities was obtained by 5×10^{-7} M Hg^{++} and 5×10^{-5} M Hg^{++}, respectively. Similar results were obtained with Ag^{+}, Cu^{++} and parachloromercuribenzoate (p-CMB, Table II).

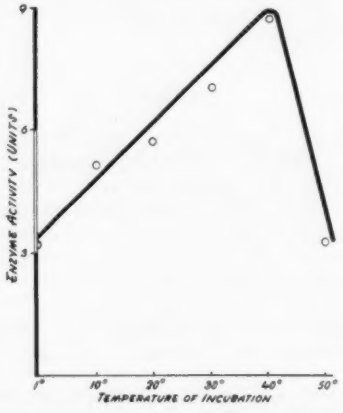

FIG. 3. THE EFFECT OF TEMPERATURE ON THE ACTIVATION REACTION. The incubation mixture consisted of 1 ml of "sensitive" hemolysate and 1 ml of stroma suspension. Six tubes were incubated for 15 minutes at the indicated temperature. After the incubation the mixture was centrifuged at 28,000 G for 10 minutes and the supernatant was tested.

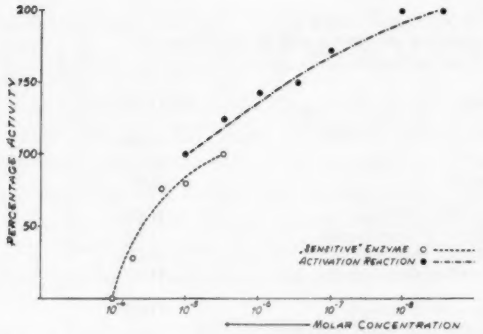

FIG. 4. THE EFFECT OF Hg^{++} ON G6PD ACTIVITY BEFORE AND AFTER ACTIVATION. The data are expressed as per cent activation. The maximal activity of G6PD prior to activation is referred to as 100 per cent. Since after activation the level of activity was twice the original level, it is referred to as 200 per cent. Samples of 1 ml "sensitive" hemolysate (○---○) and activation reaction (●---●), which consist of 1 ml sensitive hemolysate and 1 ml stroma suspension, were incubated with different concentrations of Hg^{++} at 37°C for 30 minutes. After incubation the samples were spun down at 28,000 G for 10 minutes, and G6PD was assayed on the supernatant.

The action of the activator on partially purified enzyme was also tested; 0.5 ml of stroma suspension was added to 0.5 ml of partially purified enzyme solution which contained 0.045 Δ A per minute per 0.5 ml. After 15 minutes, incubation the Δ A increased to 0.100 Δ A per minute.

The enzyme obtained from three normal hemolysates could not be activated under the same experimental conditions.

The effect of various amounts of stroma on the activation of G6PD is given in Table III. As can be seen, maximal activation was obtained in 15 minutes by 1 ml of normal stroma (30 mg dry weight) added to 1 ml hemolysate. Smaller amounts of stroma did activate the sensitive hemolysate to the same level in a longer period of time. Larger amounts of stroma did not augment the G6PD activity in sensitive hemolysates more than 1 ml of stroma. Furthermore, the ability of the stroma to activate G6PD remained practically unchanged after four successive activations, using the same stroma on different samples of sensitive hemolysates (Table IV).

TABLE II

The effect of inhibitors on G6PD and the activation reaction

	I_{50} values*		
	Cu	Ag	p-CMB
G6PD activity	3×10^{-4}	1×10^{-4}	5×10^{-5}
Activation reaction†	7×10^{-5}	1×10^{-5}	5×10^{-6}

* I_{50} represents the molar concentration that causes 50 per cent inhibition of the reaction.

† The activation reaction which consisted of 1 ml of hemolysate to 1 ml of stromal suspension was incubated with the Cu^{++}, Ag^{+}, and p-CMB in different concentrations for 15 minutes at 37°C. After centrifugation for 10 minutes at 28,000 G, the G6PD was assayed in the supernatant.

The activator was found to be labile; it was destroyed when an acetone-dried powder was prepared from the stroma or by freezing and thawing the stroma. It was also completely destroyed by heating the stroma for 10 minutes at 80°C, and partially destroyed at lower temperatures (Figure 5).

G6PD activity in red cell stroma. During our preliminary work on the activating capacity of normal red cell stroma, no G6PD or 6PGD could be demonstrated in it. Normal red cell stroma was incubated with water, saline or Tris buffer, pH 7.4, for up to 2 hours and no enzymatic activity could be shown in the supernatant. However, when the stroma was tested by the method of Motulsky, G6PD and 6PGD activities were de-

TABLE III

The effect of different amounts of stroma on G6PD activation (representative experiment)

Amt. of stroma	"Sensitive" hemolysate	Time in minutes				
		0	15	30	45	60
ml	*ml*			*Δ A/min/0.1 ml*		
0.2	1	0.000	0.000	0.003	0.0064	0.010
0.3	1	0.000	0.000	0.0044	0.010	0.010
0.5	1	0.000	0.0024	0.008		0.008
1.0	1	0.000	0.010			0.010
2.0	1	0.000	0.010			

TABLE IV

A comparison between the activating capacity of normal red cell stroma and its G6PD content *

Expt. no.	Incubation mixture	G6PD activity†
1	Hemolysate alone‡	0.060
2	Stroma alone‡	0.000
3	Stroma after 5 freezings and thawings	0.032
4	Stroma + hemolysate	0.300
5	Stroma of Expt. 4 + a second aliquot of hemolysate	0.280
6	Stroma of Expt. 5 + a third aliquot of hemolysate	0.260
7	Stroma of Expt. 6 + a fourth aliquot of hemolysate	0.200
8	Stroma of Expt. 7 after 5 freezings and thawings	0.020
9	Stroma after 4 successive incubations with water followed by 5 freezings and thawings (control of Expt. 8)	0.030

* All G6PD assays were performed on the supernatant after 10 minutes' centrifugation at 28,000 G.
† In Δ A per minute per 1 ml stroma or hemolysate.
‡ In all the experiments "sensitive" hemolysate and/or normal stroma were used; 1 ml of each was incubated for 15 minutes at 37° C prior to G6PD assay. Aliquots of the same stroma suspension and the same hemolysate were used in all the experiments.

tected in normal red cell stroma and on 6PGD activity in sensitive stroma. In normal stroma the change in color from blue to red appeared after less than 2 hours. In sensitive stroma no discoloration was observed after 12 hours when glucose-6-phosphate was used. When 6-phosphogluconate was used, the change in color appeared after less than 2 hours, as in normal stroma. In view of this finding, the possibility arose that the effect of normal stroma on sensitive hemolysate could be the result of G6PD elution from the stroma rather than a reaction from the activation.

The following experiments were performed to elucidate this issue. One ml stroma suspended in water was frozen up to 10 times and the supernatant was tested for G6PD and 6PGD activities; the mean of five experiments is presented in Table V. When the amount of stroma was doubled or tripled, the amount of enzyme released upon freezing also doubled or tripled. In Table IV we compare the activating capacity of 1 ml of stroma to its G6PD content. As can be seen from this table, the amount of enzyme released from normal stroma was found to be much smaller than the increase of G6PD activity in a sensitive hemolysate incubated with normal stroma. The same stroma on four consecutive activations increased the G6PD activity of sensitive hemolysate about 30 times more than the amount of enzyme that could have been released from this amount of stroma by freezing and thawing. Furthermore, when the same stroma was frozen and thawed, the amount of G6PD released was not much lower than the amount of enzyme that was present in the stroma before activation (Table IV). In addition, G6PD could not be extracted from stroma suspended in 1 M or 2 M NaCl solution. These results prove that the increase of G6PD activity in sensitive he-

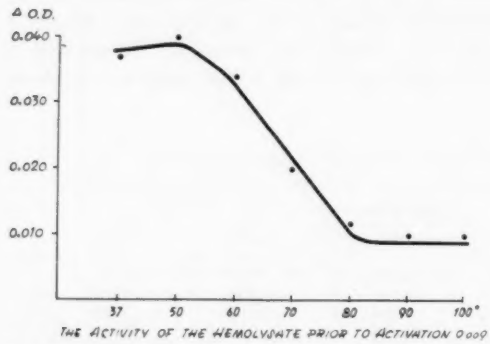

FIG. 5. THE EFFECT OF HEATING ON ERYTHROCYTE STROMA PRIOR TO THE ACTIVATION REACTION.

TABLE V

G6PD and 6PGD activities in red cell stroma *

Material	Amount	G6PD	6PGD
	ml	*Δ A/min/ml stroma*	
Normal stroma	1	0.040	0.030
"Sensitive" stroma	1	0.008	0.032

* One ml of the respective stromas, containing about 30 mg dry weight, was frozen and thawed 10 times. The sample was centrifuged at 28,000 G for 10 minutes and the supernatant was used for enzymatic assay.

molysates upon the addition of normal stroma was indeed an activation reaction and not an elution of enzyme from stroma.

As far as the bound G6PD and 6PGD are concerned, the question arose of whether these are the same enzyme as that in the hemolysate. The pH optimum and temperature lability were found to be the same for the bound and soluble G6PD. Since these data were obtained on crude preparations, no final conclusions can yet be drawn.

DISCUSSION

The main defect in the erythrocytes of individuals "sensitive" to primaquine and other drugs as well as to fava beans was considered to be the low glucose-6-phosphate dehydrogenase activity (1, 2). This enzymatic defect was found in Negroes, non-Ashkenazic Jews, Sardinians, Sicilians, Chinese and others (10–12). This defect is transmitted as a sex-linked incompletely dominant trait with variable expressivity in the heterozygous female (13, 14). In "sensitive" Jews and Italians the enzymatic defect was found in other tissues also, while in Negroes the defect was found solely in the erythrocytes (8, 15–18).

Recently we observed that normal red cell stroma was capable of partial restoration of the enzymatic activity of sensitive hemolysates (5); this finding could suggest that the enzymatic deficiency in those individuals is of only secondary nature, while the primary defect is possibly in the stroma. This suggestion is strengthened by the fact that several authors were unable to show any qualitative difference between the G6PD of normal and sensitive erythrocytes (3, 4).

Since we demonstrated that there are stroma-bound G6PD and 6PGD, the major problem was to exclude the possibility that the activation effect was not the result of elution of the enzymes from the stroma. The following results confirm our earlier observations (5).

a) The augmentation in the G6PD activity was observed only when normal stroma was incubated with a sensitive hemolysate. Normal stroma had no effect on a normal hemolysate; in fact, it frequently decreased its activity. *b*) G6PD could not be eluted from stroma incubated at 37° with water, saline, or Tris buffer. *c*) The activating capacity of stroma did not depend upon the amount of stroma. Small amounts of stroma activated as well as large amounts, the difference being only in the rate of the reaction (Table III). *d*) The activating capacity of normal red cell stroma was found to be out of proportion to its G6PD content, as shown in Table VI. *e*) The activating capacity of frozen stroma was destroyed, while such stroma showed G6PD activity by the method of Motulsky.

These results prove that the effect obtained by normal red cell stroma on sensitive hemolysates is the result of G6PD activation. The behavior of the activation reaction—namely its temperature, pH and time-dependence as well as the capacity of the same stroma to activate successive samples of sensitive hemolysate and a sensitive partially purified G6PD—suggested its being an enzyme-enzyme activation similar to the activation of chemotrypsinogen by trypsin or of phosphorylase by phosphorylase-activating-enzyme (19, 20). However, from the data mentioned it is not yet possible to suggest a definite mechanism of the G6PD activation.

The possibility that sensitive stroma possessed an inhibitor of the G6PD activator was excluded, since sensitive stroma did not inhibit the activating capacity of normal red cell stroma on sensitive hemolysates.

The effect of heavy metal ions and p-CMB on this reaction suggests that sulfhydryl groups may be important in this reaction. Only after a highly purified G6PD is obtained, will it be possible to learn more about the specificity of the activator. Difficulties arose when we tried to purify the activator. Since it was a labile substance, it was destroyed by freezing and thawing of the stroma, by heating the stroma to 80° C, and when an acetone powder of stroma was prepared.

Our present hypothesis is that G6PD is synthesized in an inactive form in both normal and sensitive subjects. In the normal subject the enzyme is activated by an erythrocyte stroma factor. Since such an activator was found to be absent in sensitive red cells, lack of it may be responsible for the apparently low or deficient G6PD activity. In the normal erythrocytes the activator probably activated the G6PD to a maximal level *in vivo*, since the addition of normal stroma to normal hemolysate did not increase the enzymatic activity. This hypothesis will not change the genetic aspects of this abnormality, the only implication being that the primary genetic lesion affects the

G6PD activator, rather than the enzyme per se. Using this hypothesis, heterozygous females with varying levels of this enzyme are being investigated.

SUMMARY

1. A factor capable of activating glucose-6-phosphate dehydrogenase of "sensitive" erythrocytes was found in normal red cell stroma. This activation reaction was found to be dependent upon time, temperature and pH. The inhibitory affects of Ag^{+}, Hg^{++}, Cu^{++} and parachloromercuribenzoate were studied. The kinetics of the activation reaction suggest that it is enzymatic.

2. This activator was destroyed by freezing and thawing, heating, and cold acetone treatment.

3. Small amounts of tightly bound glucose-6-phosphate and 6-phosphogluconic dehydrogenase were detected in red cell stroma.

4. The data prove that the glucose-6-phosphate dehydrogenase activation is not the result of elution of the enzyme from normal red cell stroma.

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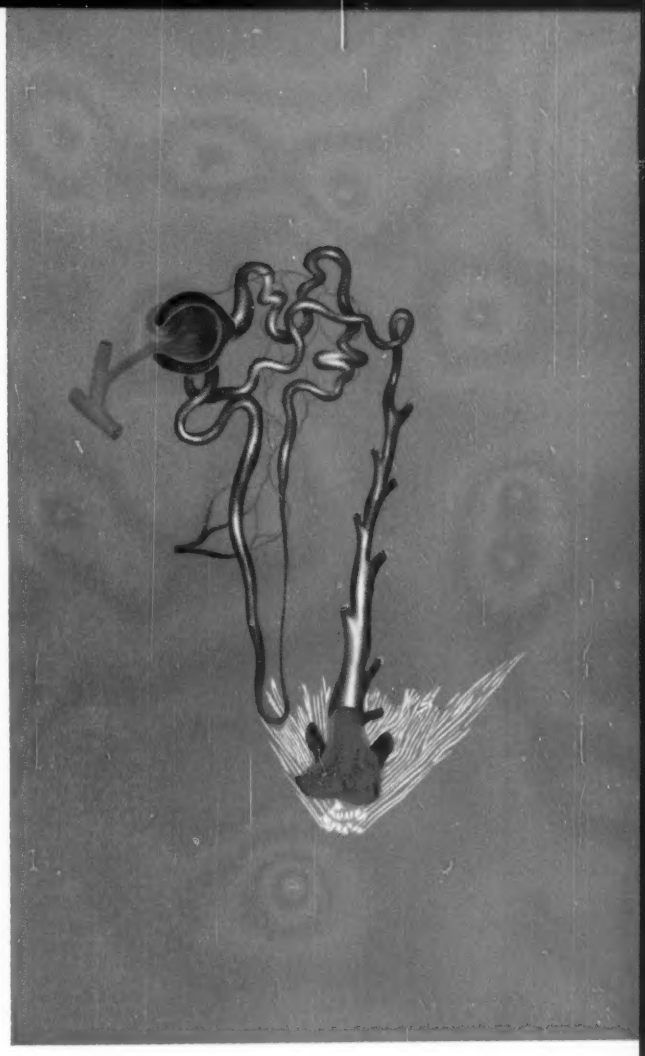

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